

UNIVERSITE DE GENEVE

Département de biologie végétale

Microbiologie

Institut de microbiologie (Faculté de médecine)

FACULTE DES SCIENCES

Professeur R.H. REGAMEY

Professeur G. TURIAN

**STRUCTURE-FUNCTION RELATIONSHIP
OF THE PATHOGENESIS OF TETANUS :
ACTIVITY OF THE TOXIN
AT THE MEMBRANAR LEVEL**

THESE

présenté à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de docteur ès sciences biologiques

par

Jessie M. ZIMMERMAN

de

COLORADO SPRINGS (Colorado, USA)

Thèse No 1829

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Editions Médecine et Hygiène

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La Faculté des sciences, sur le préavis de Messieurs R. Regamey, professeur ordinaire et directeur de thèse (Faculté de médecine), G. Turian, professeur ordinaire et responsable devant la Faculté (Département de biologie végétale), J.-P. Bargetzi, professeur ordinaire (Département de biochimie) et J.-C. Piffaretti, chef de travaux (Faculté de médecine), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 2 août 1977

Le doyen :
Bernard Levrat

Thèse No 1829



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TO MY MOTHER AND MY CHILDREN

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RESUME

Relations structure-fonction dans la pathogénèse du tétanos :
activité de la toxine au niveau de la membrane.

PREMIERE PARTIE - Interaction de la toxine tétanique et de l'anatoxine avec
des cellules en culture de Neuroblastoma : analyse par
immunofluorescence.

La liaison à la membrane de la toxine tétanique et de l'anatoxine a été étudiée avec des cellules entières de Neuroblastoma de souris (lignée C1300, clone NB2A). Des cultures mono-couche de 24 heures ont été incubées en présence de 20 Unités de Fixation (Binding Units : BU) de toxine ou d'anatoxine tétanique (une unité de fixation équivaut approximativement à 1 Lf). Les cellules ont été cultivées dans des boîtes de Pétri contenant des lamelles de verre, à 37° C et sous une atmosphère de 8 % de CO₂. Les échantillons prélevés ont été fixés à l'acétone, les cellules colorées selon la méthode de Giemsa et les antigènes fixés mis en évidence par immunofluorescence indirecte avec un anti-sérum contenant des anticorps conjugués au FITC. Les préparations ont été observées par microscope aux UV, et par microscope au contraste de phase pour l'examen de la morphologie cellulaire. Pendant une période de 24 heures et plus, une quantité croissante de toxine se fixe sur les cellules, tandis qu'aucune liaison de l'anatoxine ne peut être détectée par immunofluorescence. L'exposition à toxine de cultures en phase de croissance (présence de sérum) ou en phase de différenciation (absence de sérum) montre une nette différence dans la répartition de la fixation. Les observations faites sur les cellules traitées à la toxine ont montré, en absence de sérum, une modification importante de la morphologie cellulaire, tandis que, pour les cellules en croissance, aucun changement n'est décelable, que ce soit dans la morphologie cellulaire ou dans le nombre de cellules. L'influence de modulateurs chimiques des membranes (poly-D-glutamate, NH₄Cl, neuraminidase de V. cholerae, β -galactosidase de E. coli) a été analysée.

Ces études préliminaires ont montré que la méthodologie employée était utilisable avec succès, non seulement pour mettre en évidence et évaluer la distribution et le caractère des récepteurs à la surface cellulaire, mais aussi pour soutenir qualitativement les expériences de liaison de la toxine marquée au I¹²⁵.

DEUXIEME PARTIE - Interaction de la toxine tétanique et de l'anatoxine avec des cellules en culture de Neuroblastoma : étude de la liaison de la toxine et de l'anatoxine marquées au I^{125} .

Nos précédentes recherches, basées sur des techniques d'immunofluorescence, ont montré que la toxine tétanique se fixe, dans des cultures de tissus, aux cellules de Neuroblastoma de souris (lignée C1300), qu'elles soient en phase de croissance ou de différenciation. Nous avons confirmé ces résultats en utilisant la toxine et l'anatoxine tétaniques marquées au I^{125} . Nous n'avons pas détecté de liaison pour l'anatoxine. Les expériences de fixation de la toxine à des cellules en phase de croissance montrent deux niveaux d'affinité pour la liaison dépendant des résidus "acide sialique" : un type de liaison est déplaçable par la toxine native, l'autre pas. Il n'y a pas de fixation décelable en présence de neuraminidase ou de β -galactosidase ; de plus, ces deux enzymes sont capables d'enlever totalement la toxine- I^{125} fixée, lors d'une préincubation, aux cellules en croissance.

Des expériences analogues montrent que les cellules en phase de différenciation présentent trois classes distinctes de récepteurs : dépendant des résidus "acide sialique", dépendant de liaisons β -galactoside, et indépendant de l'action de la β -galactosidase. La première classe est sensible à la neuraminidase ; elle peut être divisée en deux groupes : déplaçable et non déplaçable par la toxine native. La deuxième classe comprend des récepteurs sensibles uniquement à la β -galactosidase, tandis que les sites de la troisième classe sont indépendants et définis par la proportion de toxine liée en présence de β -galactosidase. Nous avons étudié la cinétique d'interaction de la toxine- I^{125} aux surfaces cellulaires sous différentes conditions ; la fixation est saturable en l'espace d'une heure, quelle que soit la phase de développement des cellules. Ces résultats soutiennent l'hypothèse selon laquelle les liaisons "efficaces" et "non-efficaces" sont dues à des propriétés différentes de récepteurs distincts.

TROISIEME PARTIE - Interaction de la toxine tétanique et de l'anatoxine avec des cellules en culture de Neuroblastoma : mise en évidence in vitro d'une activité biologique de la toxine tétanique.

Lors de nos premières observations sur les cellules de Neuroblastoma (lignée C1300, clone NB2A) exposées à la toxine tétanique en DMEM (Dulbecco's Modified Eagle's Medium) sans sérum, nous avons mis en évidence la réversion de la différenciation morphologique. Dans ces expériences, nous avons utilisé d'autres méthodes pour l'induction des structures "neuroniques", dans le but de mieux déterminer la nature et l'étendue de l'activité biologique de la neurotoxine tétanique. Les essais ont été effectués en utilisant différents moyens comme pour l'induction de l'extension des structures "neuroniques" ; leur sensibilité à des inhibiteurs tels que la colchicine, la cytochalasine B et la toxine tétanique a été étudiée. Les résultats décrits suggèrent que les moyens d'induction utilisés peuvent être séparés en deux classes, d'abord en fonction de l'activité des produits utilisés, et ensuite en fonction de la capacité de la toxine tétanique de provoquer la réversion des structures "neuroniques" déjà formées. Les cellules incubées en présence de sérum additionné soit de dibutyryl-cyclic-AMP, soit d'aminoptérine, sont insensibles à la toxine tétanique. L'adjonction

de toxine à des cellules en DMEM sans sérum ou en GCCM (glial cell conditioned medium) aboutit à la réversion de la différenciation morphologique. Il n'a pas été effectué d'expériences permettant de définir les cellules différenciées du point de vue biochimique.

Les résultats obtenus à l'aide de GCCM et de DMEM sans sérum montrent que des concentrations élevées de toxine aboutissent, en 4 heures, à une réversion différenciée des structures "neuroniques". Ces dernières, à une concentration donnée de toxine, se rétractent d'une grande longueur à une plus courte ; cependant, en GCCM, il n'y a pas de disparition complète des structures "neuroniques", quelle que soit la concentration en toxine. Etant donné qu'en DMEM sans sérum, la concentration critique est bien plus faible, cela confirme l'effet d'une substance active dans le GCCM.

Avec des concentrations de toxine plus faibles, il faut 24 heures pour obtenir des résultats semblables. Ceci suggère qu'aux fortes concentrations de toxine, il faut 4 heures pour que puisse s'accumuler la quantité critique nécessaire à la réversion, tandis qu'il faut 24 heures aux faibles concentrations. Ces résultats infirment aussi l'hypothèse selon laquelle la cause de la réversion serait une modification de la membrane.

Les études de liaison avec la toxine marquée au I^{125} montrent que la fixation se fait quel que soit le mode d'induction des cultures. La sensibilité à la neuraminidase et à la β -galactosidase varie en fonction de la liaison "efficace" ou "non-efficace".

PART I

INTERACTION OF TETANUS TOXIN AND TOXOID WITH CULTURE NEUROBLASTOMA CELLS : ANALYSIS BY IMMUNOFLUORESCENCE

Summary. The primary interaction of tetanus toxin and toxoid with mouse neuroblastoma cells (C1300, clone NB2A) in tissue culture was studied using direct immunofluorescence. Experiments were done in standard routine cultures and also those influenced by chemical modulators.

There is a difference in the characteristic binding response between the growth culture cells (grown in presence of fetal calf serum) and differentiating culture cells (grown in absence of serum). Exposure to the toxin gives no visible effect on the cell division or viability in growth cultures ; whereas in differentiating cells the processes are shortened and the adherence to the glass is diminished without involving significant cell death. The toxoid did not bind at all under the same experimental conditions.

Since there was no biological effect in growth cultures we have called this binding INEFFECTIVE, and in the case of the differentiating cells, EFFECTIVE binding.

Stimulation of pinocytosis increases the uptake of toxin in both cultures. Presence of some surface bound toxin still remaining on the differentiating cells indicates the possibility of another sort of mechanism for internalization. Pre-treatment of the cells with neuraminidase or β -galactosidase to alter the membrane gangliosides eliminates binding in growth cultures but not in differentiating cultures.

From these results we suggest that even though the toxin may well bind to gangliosides, at least in the differentiating cultures they are not solely responsible for the fixation. The morphologically observed EFFECTIVE binding is probably that not related to gangliosides.

Key words : Tetanus toxin - Toxoid - Gangliosides - Immunofluorescence - Neuroblastoma cells.

Running title : Tetanus Toxin binding in Tissue Culture

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INTRODUCTION

In recent years, there has been considerable work done on the structure of tetanus toxin (Bizzini et al., 1973), its enzymatically derived subunits (Craven and Dawson, 1973 ; Helting and Zwisler, 1974 ; Matsuda and Yoneda, 1974), the distribution of toxin in vivo (Kryzhanovskii, 1973 ; Habermann and Dimpfel, 1973 ; King and Fedinec, 1974) and the retrograde intraaxonal transport (Erdmann et al., 1975 ; Price et al., 1975 ; Stöckel et al., 1975). There has seemingly been no direct evidence showing the binding of the toxin to cell surface receptors. Since the first step in the pathogenesis of tetanus may be the interaction of the toxin with the cell membrane, the following qualitative studies were conducted using fluorescent techniques in an attempt to obtain a general view of the proposed binding. Parallel studies were undertaken using the toxoid. Mouse neuroblastoma cells were selected as substrate due to their capacities : (i) to elaborate neurotransmitters (Augusti-Tocco and Sato, 1969 ; Breakfield, 1976), (ii) to generate action potentials in response to electrical stimulation in vitro (Nelson et al., 1969) and (iii) to produce neuronlike processes after a decrease in serum concentration of the culture medium (Seeds et al., 1970). These responses, simulating an in vivo situation, make these cells ideal "laboratory animals" to test a toxin which affects only the nervous system. Thereby, it also offers the possibility of determining a relevant physiological activity of the toxin at the cellular level which might serve as a useful tool in understanding the basic mode of action at the clinical level.

In these preliminary binding assays, the fluorescent techniques have not only proven useful as a method to visualize, and a probe to evaluate, the distribution of cell surface receptors and their character, but serve also as a qualitative back-up to (^{125}I)-toxin binding experiments (in preparation).

MATERIAL AND METHODS

Purified tetanus toxins were gifts of Dr F. Massone, Instituto Sieroterapico e Vaccinogeno Toscano "Sclavo", Siena, Italy ; Dr P. Legler, Schweiz. Serum- und Impfinstitut, Bern ; Drs Dietzel and Zwisler, Behringwerke AG, Marburg/L., Germany ; Dr A. Turpin, Institut Pasteur, Garches, France ; The Wellcome Research Laboratories, Beckenham, U.K. ; Prof. D. Ikić, Institute of Immunology Zagreb, Yugoslavia ; Dr I. Joó, "Human" Institute for Serobacteriological Production and Research, Budapest, Hungary ; Dr R. Triaux, Institut Mérieux, Lyon, France. Tetanus Antitoxin (Tetuman) and Toxoid (Anatoxal) were from Berna. Mouse Neuroblastoma C1300 cells (clone NB2A) were a gift of Dr D. Monard, Friedrich-Miescher Institut, Basel ; Dulbecco's Modified Eagles Medium (DMEM) was obtained from Gibco and Fetal Calf Serum (FCS) from Flow Laboratories. Fluorescein isothiocyanate (FITC), poly-D-glutamate (PDG), neuraminidase (V. cholerae) (EC. 3.2.1.18) and β -galactosidase (E. coli) (EC. 3.2.1.23) were purchased from Sigma. Photographs were taken with a Zeiss microscope using GAF 500 (Anscochrome) color slide film. All reagents were laboratory grade.

METHODS

FITC conjugation : FITC conjugated toxin and antitoxin were prepared according to the method of Thé and Feltkamp (1970) as described in the Handbook of Experimental Immunology (1973). After gel filtration on Sephadex G25, conjugated protein was frozen in 2 ml aliquots until used. Activity was titrated against standardized toxin of 1000 Lf per ml. FITC-conjugated toxin retained full biological activity when assayed in mice.

Tissue culture : Cells were grown in 60 mm plastic Petri dishes containing 18 mm square coverslips. They were cultivated in DMEM + 0.2 % bicarbonate + 10 % FCS, and incubated in 8 % CO₂ + 92 % air humid atmosphere at 37°C. Cells to be used as growth cultures were continued in presence of serum. Differentiating cultures were stimulated after 24 h by replacing the medium with serum-free DMEM and incubated for an additional 48 h until processes were well formed.

For immunofluorescent assay, 20 Binding Units (one BU equals approximately one Lf (Limes flocculation) according to Kryzhanovski, 1975) of toxin or toxoid were added directly into the culture medium and mixed well. Incubation was continued at 37°C until samples (coverslips) were removed at 10, 30, 60, 120 min and 24 h.

Coverslips were rinsed for 5 min in ice-cold phosphate buffer (0.15 M pH 7.4) then fixed in acetone at -60°C for 20 min. Samples were stored in the cold until used. Pre-incubations with modulators were carried out for 2 h using 1 mM NH₄Cl or 0.1 % poly-D-glutamate ; with 0.1 % neuraminidase and β -galactosidase, the pre-treatment was for 30 min, then the cultures were washed in fresh medium before the toxin-containing medium was added.

Frozen coverslips were washed in Veronal Buffer (pH 8.3) and exposed to FITC antitoxin. Cells were stained with Eriochrome Black. Preparations were observed by phase contrast for morphology and under the fluorescent microscope for binding evidence.

For direct FITC-toxin assays, the conjugated protein was added to the culture medium. For indirect immunofluorescence, the cells were treated with toxin and unlabelled antitoxin, then with FITC-Anti-Human-Immunoglobulin.

RESULTS

Morphological Observations. The cells which had been exposed to toxin and toxoid were observed at intervals under an inverted microscope. Size, shape, population and process formation were considered. After 48 h, marked differences became evident (Table 1). Since there was a visible change for all named criteria in the differentiating cultures, and none in the growth cultures, the toxin interactions were termed Effective and Ineffective respectively. In presence of toxoid, there was no change in either form of culture. Until a precise measurable physiological response is found, the morphological changes serve as indications of biological effect. The growth cultures to which the toxin was added showed the same characteristics as the control cultures. Our results show that part of the

reaction in the differentiated cell culture, toxin binding leads to contraction of processes which is accompanied by a diminished adhesion to the glass. Tested for Trypan Blue exclusion, less than 10 % of the free cells took up the dye. When the floating cells were reseeded into fresh medium with FCS, they grew as normal growth cultures. The population reduction is therefore not directly due to cell death.

Toxin Binding Revealed by FITC-Antitoxin. It has been demonstrated that toxin neutralized with antitoxin is still able to bind to tissue, and also that fixed toxin can still react with antitoxin (Kryzhanovski, 1973). These data indicate that the fluorescent antibody technique is applicable to these studies and enable us to probe for membrane-bound antigen. The surface bound toxin appears as patches or aggregates shown by the large spots of fluorescence (Figure 1). No evidence of capping was observed. After 60 min, some toxin appears to be internalized, more so in the differentiating cultures than in the growth cultures.

Stimulation of Pinocytosis. Cohn and Parks (1967) have shown that poly-D-glutamate stimulates pinocytosis and vesicle formation in tissue culture. After 60 min of exposure to the toxin following 2 h pre-incubation with 0.1 % poly-D-glutamate, the fluorescence is seen inside the cell. Fluorescence is seen entirely within the cells in the growth cultures, whereas in the differentiating cultures membrane-bound toxin is still evident in addition to that internalized (Figure 2). No morphological changes were seen at this time in the differentiating cultures when observed under the inverted microscope. When the remaining toxin was taken up, processes retracted and the cells began to float off the glass.

Modulation of Cell Surfaces. a) Ammonium chloride solubilizes surface cAMP-independent protein kinases which are responsible for the phosphorylation of exogenous substrates (Rubin et al., 1972). Ivins et al. (1975) showed that $1 \times 10^{-3} \text{M}$ NH_4Cl rendered $\text{HEp}-2$ monolayers insensitive to the action of diphtheria toxin. It did not, however, inhibit the surface interaction of the toxin with the membrane. We have used a similar treatment with the neuroblastoma cells, in the growth and differentiated forms, to study the effects on binding of tetanus toxin. In the growth cultures, there is still binding, indicating that the salt has no notable effect under these conditions (Figure 3). However, in the differentiated cultures, there is no evidence of binding, thus substantiating the argument that there is a difference in the relationship toxin-membrane in the two types of cultures.

b) VC neuraminidase (sialidase) removes about 20 % of the sialic acid residues from the cell surface, thus transforming ganglioside types GGnSSLC and SGGnSSLC (van Heyningen, 1971) (or GD1_b and GT1 by the Svennerholm notation (1963)) to GGnSLC (GM1). Since these are the two gangliosides proposed as receptors for tetanus toxin (van Heyningen, 1974), treatment with this enzyme was assayed in our system. In the growth cultures the binding was totally eliminated. The differentiating cultures on the other hand showed diffuse "halo" binding resembling the glow seen in indirect immunofluorescence preparations using FITC-Anti-Human-Immunoglobulin (Figure 4). This suggested that the patching seen in non-treated (i.e. non-neuraminidase treated) preparations may be related to ganglioside binding.

c) We then examined the role of the ganglioside terminals per se. β -galactosidase shears off the ganglioside at the glucose residue (Craven et al., 1965). After 30 min pre-incubation, there was still pinpoint binding in the differentiating cultures, but none at all in the growth cultures (Figure 5). Control experiments were performed with FITC-Cholera-Toxin which binds to GM1 (Cuatrecasas, 1973). We confirmed an increase in binding after neuraminidase treatment, found by Haskar et al. (1974). There was total suppression of binding in our system after β -galactosidase treatment.

Toxoid Binding. We repeated the same basic binding studies using tetanus toxoid. Regardless of concentration used there was no demonstrable binding using the fluorescent techniques. To verify this (^{125}I)-toxoid was assayed (manuscript in preparation). Mouse neuroblastoma cells bind tetanus toxin, but they do not bind toxoid.

DISCUSSION

Using fractions of homogenized tissue, Kryzhanovski (1975) has shown sub-cellular fixation of tetanus toxin and toxoid. Habermann (1973) demonstrated tissue fixing of (^{125}I)-toxin and toxoid, the latter only in very elevated concentrations. One of the purposes of the present investigation was to establish an in vitro tissue culture system with which to attempt a correlation with in vivo studies. Mouse neuroblastoma cells showed the most promise for such a relationship. Immunofluorescent procedures are relatively simple, yet many interesting conclusions can be approached with them. The fact that the toxin binds to cell membranes and the toxoid does not, sheds light on the effect of toxoidization on the toxin molecule. We suggest that during the process of toxoidization, the discriminator factors on the toxin molecule are distorted, disfigured or destroyed such that the receptors on the membrane used for fixation no longer recognize them. Since other work (Kryzhanovski, 1975) has shown that there is competition and displacement between the toxin and toxoid at the sub-cellular level, this intimates that a secondary binding locus for intracellular activity is probably still intact in the toxoid. Toxoidization is thereby due to primary site disruption involving binding and not detoxification per se. Kryzhanovski (1973) has proposed that the toxin molecule has at least three groupings : one of them for the binding on the receptor, a second for reaction with antitoxin and the third for the specific toxic activity. Resulting from our findings in tissue culture, we propose that within the receptor binding grouping there are at least two binding sites on the toxin molecule, corresponding to EFFECTIVE and INEFFECTIVE sites on the surface membrane. We suggest that a toxin molecule could be bound at either of the sites, but not at both simultaneously. INEFFECTIVE binding is defined as toxin bound to that site which produces no visible biological effect, as in growth cultures. It is sensitive to β -galactosidase and neuraminidase and demonstrable by fluorescent and immunofluorescent techniques. EFFECTIVE binding, on the other hand is toxin bound to a site which, as in the case of differentiating cultures leads to a visible biological effect. It is not sensitive to β -galactosidase or neuraminidase and is demonstrable with the fluorescent and immunofluorescent techniques. The re-partition of these two sites on differentiated cell membranes will have to be determined by the quantitative (^{125}I)-toxin studies. Since the retraction of pro-

cesses followed this binding when the ganglioside-dependent binding had been eliminated, we link the ganglioside-independent binding to the biological effect. Further support for this is found in the fact that tetanus toxin-antitoxin complex exposure of the cells led to no process retraction after cells had been treated with β -galactosidase. Since there was no visible uptake under these conditions the biological effect can also be suggested to be linked to the uptake of the toxin.

A toxin molecule bound to the EFFECTIVE site on the cell membrane would be destined for internalization and transport to the locus of action to cause the biological (and physiological) effect. The ultimate toxin effect would be dependent upon the amount bound to EFFECTIVE binding sites, the lag time before uptake and the rate of internal transport. The lag time must be fairly long before the Minimum Toxic Dose is taken up since in tissue culture the differentiated cells do not show retraction of processes and lack of adherence before about two days have passed. The INEFFECTIVE-site-bound toxin would be blocked by this binding, as we have shown in our experiments with growth culture cells, taken up in a physiologically insignificant manner leading to enzymatic degradation in cellular lysosomes as are other foreign proteins (e.g. albumin and histones) (Ryser, 1958).

From our findings in these experiments with neuroblastoma C1300 (clone NB2A) there is a difference in response to exposure to tetanus toxin in growth and differentiated cultures. In the growth cultures, the toxin binds and is taken up but with no apparent effect on the multiplication of the cells. Stimulation of pinocytosis augments the rate of uptake, and after 60 min there is no longer any surface binding present. Exposure of the growth culture cells to antigen-FITC-antibody complex showed no internalization of fluorescence even with pinocytotic stimulation. In the differentiating cells, however, the interaction of the cells with tetanus toxin is visibly effective in reducing the population and limiting the length and frequency of process formation. Our results show that even with pinocytotic stimulation by poly-D-glutamate, not all of the surface-bound toxin is taken up; a small amount remains on the membrane. We therefore speculate that there might be another mechanism for its internalization. Very little uptake was demonstrated in growth cultures without poly-D-glutamate stimulation; in contrast almost complete uptake occurred after treatment. The INEFFECTIVE uptake appears to be of a pinocytotic nature, since there was still no visible effect upon the cells.

Removal of the sialic acid residues by neuraminidase treatment prevents the toxin binding to growth culture cells. Moreover, the lack of inhibition of tetanus toxin binding on differentiated cells points toward a sialic-acid related binding as the INEFFECTIVE variety which is however perhaps common to both types of cultures. On the other hand, in the differentiating cell cultures, binding is still present after both neuraminidase and β -galactosidase treatments. The binding after neuraminidase treatment is diffuse, not patchy as in untreated cells. This may reflect properties of the receptor with respect to mobility within the membrane. The binding of the toxin-anti-toxin complex as demonstrated with FITC-Anti-Human Immunoglobulin presents the same aspect, indicating again the lack of mobility of the receptors as induced by the antiserum. Other studies of the relationship of gangliosides to tetanus toxin binding (van Heyningen, 1959, 1971,

1974) have shown that with a ratio of 3×10^9 mols of ganglioside to 1 mol of toxin the activity could be greatly reduced but not entirely eliminated. If the tetanus toxin molecule possesses multiple membrane bindable units, one or more of these units could fix to a ganglioside as INEFFECTIVE binding ; another, not related to the ganglioside is correlated with EFFECTIVE binding and linked to the subsequent uptake and pathogenesis of the disease. In a wound, toxin produced by the bacterium (Clostridium tetani) would arrive at the membrane and could be bound by either of the receptors. Thus the number of molecules of toxin in a Minimum Lethal (or Intoxicating) Dose is not an indication of the number of sites on the membrane which are EFFECTIVE in producing physiological effect.

With this proposition of the possibility of multiple cell receptor binding, we suggest more than one possibility for an uptake mechanism : INEFFECTIVE by pinocytosis and EFFECTIVE by perhaps another mechanism. Bonventre et al. (1975) have demonstrated this possibility using diphtheria toxin. Ammonium chloride did in fact inhibit uptake and maintained most of the toxin at the membrane ; however, it did not negate cell-toxicity. The protein synthesis was inhibited though most of the toxin was either still on the membrane or in lysosomes inside the cells. They pointed out that if pinocytosis were not the physiologically significant mechanism for uptake then another mechanism might be operative. In the case of tetanus toxin, this remains highly speculative until a measurable relevant physiological activity is found to test the hypothesis.

CONCLUSIONS

The evidence presented here indicates the existence of two sorts of binding which we have called EFFECTIVE and INEFFECTIVE. We conclude that the EFFECTIVE binding is not ganglioside-dependent and that pinocytosis is not necessarily the pathway for the EFFECTIVE uptake. INEFFECTIVE binding is ganglioside-dependent. Further studies are in progress to identify the nature of the proposed EFFECTIVE receptor and the mechanism of transmembration (i.e. direct passage across the membrane) of the toxin.

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TABLE I

Morphological Observations

Culture	Control	+ Toxin	+ Toxoid
Growth (+ FCS)	Confluent, +++ population, rounded cells	No change	No change
Differentiating	Population ~60 % of that of growth cultures. Kite- shaped large cells with long proces- ses, several per cell	Population dimi- nished by 75 %, short processes on smaller oval cells, usually 2 per cell ; floating live cells	No change

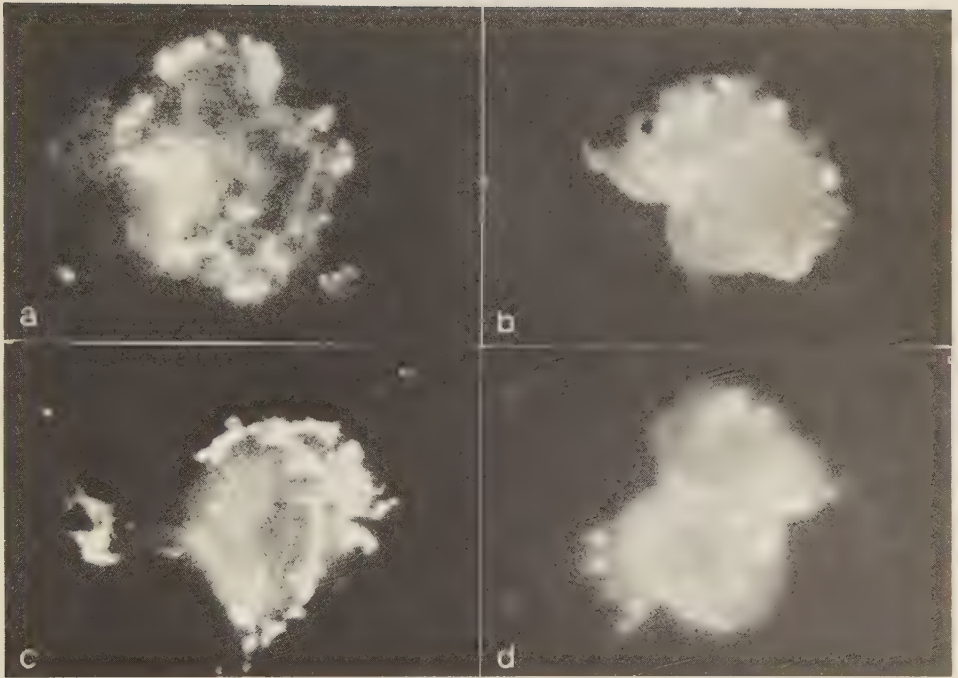


Fig. 1 : Enlargements of photomicrographs (original magnification 500 x) which were taken with a Zeiss microscope incorporating a photo device, in color (Anscochrome 500 ASA GAF film). The full impact of the contrast cannot be demonstrated in the monochromatic renditions that we are restricted to in this presentation. a) Growth culture cell after exposure to tetanus toxin for 60 min. The bright patches on the surface are interpreted to be aggregates of toxin due to migration within the membrane of the receptor-toxin complex, revealed by FITC-Antitoxin. The subdued fluorescence is assumed to represent internalized toxin. b) Surface patches are still visible after 24 h and the internalized fluorescence appears concentrated in one area. c) Differentiating cell shows relatively more large spots of external fluorescence after 60 min as well as a patch on a short process. d) After 24 h, the differentiated cell shows proportionally more subdued, internal fluorescence, with some remaining on the surface.



Fig. 2 : Cells pre-exposed to poly-D-glutamate and continued during incubation with toxin. a) After 60 min, the growth culture cells have completely internalized the toxin, fluorescence is all inside the cell. b) The differentiated cells, on the other hand, still show bright surface spots after 60 min as well as that inside the dark, nuclear area. c) After 24 h, the differentiating cell has totally internalized the antigen and shows a bright halo around the two large nuclei. The fluorescence inside the nuclei remains unexplained.

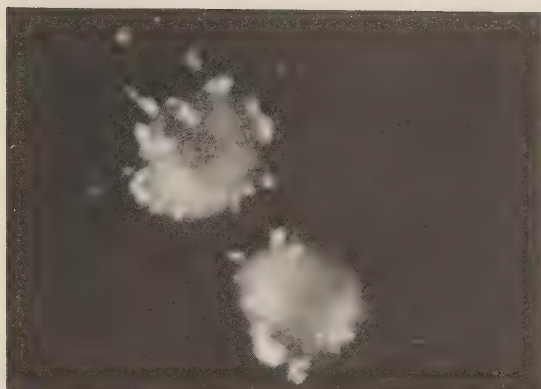


Fig. 3 : Ammonium chloride treated growth culture cells show large surface patches, some subdued fluorescence seems to be internal. In the differentiating cultures, the binding was totally suppressed.

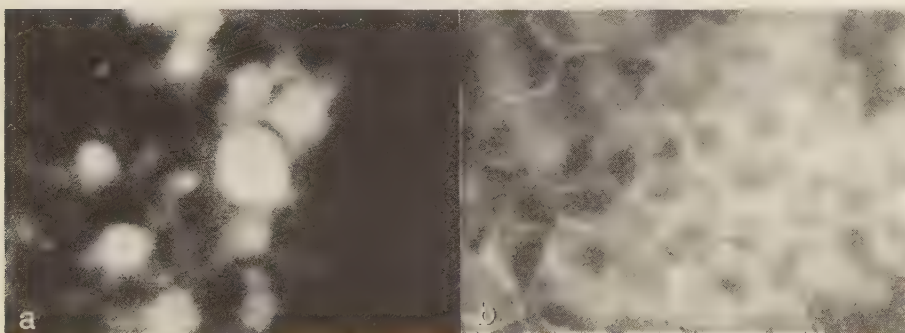


Fig. 4 : a) Indirect immunofluorescence using Anti-Human-Immunoglobulin. The whole cell glows. The toxin and antitoxin were added as a complex, thus this appears to have inhibited patch formation. b) Differentiating cultures which were exposed to neuraminidase before toxin was added. The halo, diffuse direct immunofluorescence indicates a lack of migration of antigen-receptor complex on the membrane. The growth culture cells showed no binding at all.

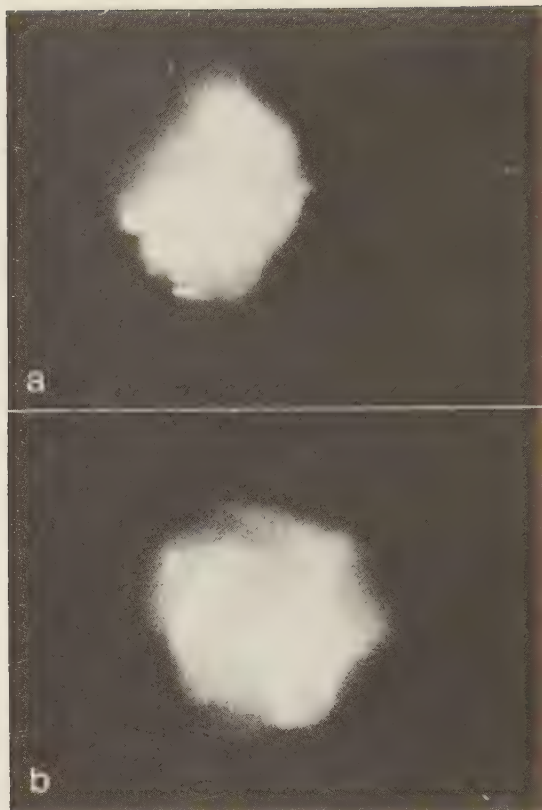


Fig. 5 : After pre-treatment with β -galactosidase a) and b) show small spots of fluorescence on differentiating cells. They are less abundant and in smaller aggregations. The contrast is less evident in monochromatic renditions than in color prints where the cells are in another color and the bright yellow-green fluorescent spots stand out.

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PART II

INTERACTION OF TETANUS TOXIN AND TOXOID WITH CULTURED NEUROBLASTOMA CELLS : ^{125}I - TOXIN AND TOXOID BINDING STUDIES

SUMMARY

(^{125}I)-tetanus toxin binds to Neuroblastoma C1300 (clone NB2A) cells in tissue culture. All of the binding in growth cultures is sensitive to neuraminidase or β -galactosidase action. We attribute this binding to ganglioside receptors. Only part of the morphologically differentiated cell binding is similarly ganglioside dependent. We show in these cells three classes of receptors : sialic acid residue dependent, β -galactoside linkage dependent, and independent. (^{125}I)-toxoid does not bind at all under our experimental conditions.

KEY WORDS : Tetanus Toxin - Neuroblastoma cells - gangliosides - neuraminidase.

RUNNING TITLE : (^{125}I)-tetanus toxin binding studies.

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INTRODUCTION

Our previous investigations using immunofluorescent techniques have shown that Tetanus Toxin binds to mouse neuroblastoma C1300 cells in tissue culture under both growth and morphologically differentiated cell conditions. We reported a biological activity such that in presence of the toxin the differentiation is reversed, the processes retract (Zimmerman and Piffaretti, 1977). This is the definition of EFFECTIVE binding. There is no visible effect in growth cultures either on division or viability, thus INEFFECTIVE binding. Other work has reported that tetanus toxin has a high affinity for di- and trisialogangliosides (van Heynigen 1974). We have shown that treatment of the growth culture cells with either neuraminidase or β -galactosidase abolishes all the binding demonstrable with fluorescent techniques. Using (^{125}I) labelled tetanus toxin we have now confirmed these results and furthermore demonstrated two affinity levels for the sialic-acid residue dependent binding : one binding displaceable by a low concentration of cold toxin, and another which is not. There is not only no binding of the toxin in presence of either enzyme, but also after pre-incubation of the growth culture cells with (^{125}I)-toxin, the action of neuraminidase or β -galactosidase removes it entirely.

On the other hand, using morphologically differentiated cells there are three distinct classes of binding receptors : sialic-acid residue dependent, β -galactoside linkage dependent, and that which is sensitive to neither and thus independent. The neuraminidase sensitive class is sub-divided into two groups, displaceable and non-displaceable Class 1a and 1b respectively. Class 2 bound toxin can be removed by β -galactosidase application only, as demonstrated by the greater decline in toxin bound using that enzyme than that using the neuraminidase. Class 3 sites are independent and are defined by the proportion bound in presence of β -galactosidase. Other work has shown that retrograde intra-axonal transport of tetanus toxin was reduced a maximum of 50 % by pre-incubation of the toxin with either a mixture of bovine brain gangliosides or a purified trisialoganglioside GT1. (Stöckel et al. 1977).

The results presented here demonstrate the kinetics of the interaction of (^{125}I)-tetanus toxin under varying experimental conditions. The binding is saturable within one hour for either cell type. The results support our hypothesis that INEFFECTIVE and EFFECTIVE binding of the toxin are due to the different character of the separate receptors. No Toxoid binding was demonstrated at all in these experiments.

MATERIAL

Carrier free (^{125}I)-Na was purchased from the Radiochemical Center, Amersham, UK. Specific Activity 8-14 Ci/mg Iodine.

Neuroblastoma C1300 cells (clone NB2A) were a gift of Dr D. Monard, Friedrich Miescher-Institut, Basel. The Tetanus Toxin was a gift of Dr B. Bizzini, Institut Pasteur, Paris. Tetanus Toxoid (Anatoxal) was obtained from BERNAL, Switzerland.

Neuraminidase (V. Cholerae) (EC 3.2.1.18) and β -galactosidase (E. coli) (EC 3.2.1.23) were purchased from SIGMA. Plastic tubes were from Milian, Geneva, Glass coverslips from Auer, Bittmann and Soulier, Basel. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from GIBCO.

Fetal Calf Serum (FCS) was from Favola or Colorado Serum Company. All reagents used were laboratory grade.

Monkey Kidney (CV1) and Calf Lung cells were from stock cultures and the Chicken Erythrocytes were obtained from a laboratory chicken.

METHODS

Purified Tetanus Toxin and commercially available Toxoid were iodinated according to Greenwood et al (1963) as modified by Stöckel et al (1974). For labelling, 250-300 μg of the protein were used and 5mCi (^{125}I)Na. The specific activity of the labelled toxoid was 2.5 $\mu\text{Ci}/\mu\text{g}$ and the final concentration was about 100 $\mu\text{g}/\text{ml}$. The specific activity of the (^{125}I)-toxin was 7.95 $\mu\text{Ci}/\mu\text{g}$ and the final concentration was about 185 $\mu\text{g}/\text{ml}$. Protein concentration was determined by the method of Lowry et al (1951). A Nuclear Chicago Well Counter was used throughout these experiments.

Mouse neuroblastoma C1300 (clone NB2A) cells were grown on 12 x 32 mm glass coverslips inserted into 10ml capped plastic culture tubes. They were cultivated in DMEM + 0.12 % bicarbonate + 10 % FCS and incubated at 37° in an 8 % CO₂ atmosphere. Growth cultures were fed after 24 h and continued for 48 h in presence of FCS. A portion of the cultures were stimulated to differentiate after 24 h by replacing the medium with serum-free DMEM. These cells were incubated for two to three days until processes were fully extended and well formed. For the binding assays, 10 μ l (3.26 x 10⁵ cpm) of the diluted toxin or (1.57 x 10⁵ cpm) toxoid were added directly to the culture tube. Final concentration of the toxin was 10⁻¹²M or 25 binding units (25Lf) of the toxoid (Kryzhanovski, 1975).

After exposure at 4° C to the (¹²⁵I)-toxin or toxoid the coverslips were removed at set time intervals, washed three times in three changes of Phosphate Buffered Saline (PBS) pH 7.4, 0.15 M and placed directly into counting tubes. This "solid state" binding procedure avoids any disturbance of the cells fixed to the glass and thus the processes of the differentiated cells are still intact. The differentiated morphology can only be achieved adhering to a surface. Another advantage of this method is that there is no loss of time in manipulation by centrifugation or filtration. The first reading was possible after a total time exposure of 15 sec up to the third washing. Controls were run during each experiment after 1, 2 and 3 washings with glass coverslips with cells exposed to labelled toxin and blank coverslips exposed to the toxin in the same treatment as the test cells. Three washings were sufficient to remove all of the label idly stuck to the glass.

Cell protein was determined in triplicate cultures by the Oyama and Eagle (1973) technique, a modification of the method of Lowry et al (1951) at each stage of these experiments.

Cultures treated with the modulators were pre-exposed for 30 min to the enzyme at RT°, and it remained in the medium during the assay for binding. For enzymatic dissociation, the agent was added to fresh medium and the cells, having been exposed by preincubation with (¹²⁵I) toxin were placed in the new medium after washing. Thus there was no free labelled toxin available.

The analog (labelled toxin) was assayed in mice and found to retain about 80 % of the toxicity of the native toxin. By flocculation however we found the titre unchanged.

The mean cell protein in growth cultures per coverslip was 349 μ g (6.96 x 10⁶ cells) at the time of the experiment. For the morphologically differentiated cells it was 235 μ g (4.69 x 10⁵ cells). The protein assays were done three times, and in triplicate each test.

Reverse displacement binding assays were done by preincubation of the cells with native toxin at 4° C and after 30 min the cells were washed and labelled toxin was added to fresh medium for the directly displaceable measurement. Two other sets were done, one in which after washing the cells were exposed to neuraminidase, and the other group to β -galactosidase before adding the labelled toxin. To ascertain whether or not the iodinated toxin binds to non-neuronal (like) cells, three other cell types were tested : Monkey Kidney (CV1),

Calf Lung, and Chicken Erythrocytes. The two former were cultivated in DMEM+10 % FCS, and the erythrocytes were centrifuged and resuspended in the standard assay medium. Procedures for the kidney and lung cells were identical with those for the Neuroblastoma. The erythrocytes were exposed to the toxin in suspension, centrifuged, washed three times in PBS and the bound cpm counted.

RESULTS

Growth Cell Association Kinetics :

The amount of toxin bound reached half maximal $t_{1/2} = 22.5$ sec, with a calculated $k_1 = 3.00 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$. The total cpm bound represents 1.87×10^{-4} μg toxin per coverslip, and 5.35×10^{-7} μg toxin per μg of cell protein. The binding is saturable after 30 min (Fig. 1). There was no binding at all in presence of either neuraminidase or β -galactosidase. This signifies that 100 % of the binding is sialic-acid residue dependent (Fig. 1).

Binding was assayed in the presence of a 200 fold excess of un-labelled (native) toxin. In growth cultures, 53 % of maximal (± 1.4) control cpm was recorded (Fig. 2). This result suggests the possibility of the two types of sialic-acid dependent binding, one which is displaceable and one which is not. Since there is no binding in growth cultures in the presence of neuraminidase we have separated Class 1 sites, (sialic-acid dependent) into two groups 1(a) displaceable and 1(b) non-displaceable, probably two gangliosides. The displaceable binding represents 47 % of the total (^{125}I) toxin bound, which may be referred to as the classic "specific" binding. The $t_{1/2 \text{ ON}} = 7.5$ sec and the calculated $k_1 = 1.33 \times 10^{11} \text{M}^{-1}\text{s}^{-1}$, 1.88×10^{-4} μg toxin per coverslip and 2.5×10^{-7} μg toxin per μg cell protein.

Differentiated Cell Association Kinetics

Half saturation was reached ($t_{1/2 \text{ ON}}$) in 13 sec in the differentiated cell cultures. The calculated $k_1 = 5.33 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$. The total cpm bound represents 2.95×10^{-4} μg of toxin per cell population and 1.25×10^{-6} μg of toxin per μg of cell protein. The binding is saturable after 30 min (Fig. 1).

In the presence of neuraminidase the $t_{1/2} = 4$ min 12 sec, giving a $k_1 = 2.75 \times 10^9 \text{M}^{-1}\text{s}^{-1}$. The total bound represents 1.418×10^{-4} μg of toxin per cell population on the coverslip. This constitutes about 48 % of the maximal binding and thus shows that 52 % was sialic-acid dependent (Fig. 2).

When the (^{125}I)-toxin was bound in the presence of β -galactosidase, the $t_{1/2} = 6$ min 48 sec, and the calculated $k_1 = 1.68 \times 10^9 \text{M}^{-1}\text{s}^{-1}$. Here, 26 % of the total maximal binding was achieved. This represents 7.68×10^{-4} μg toxin per coverslip and 3.26×10^{-6} μg toxin per μg of cell protein. We consider that the receptors for this interaction are independent of sialic-acid residues and also of the β -galactoside linkages which were cleaved by the enzyme. 22 % of the total toxin fixed was attached therefore to sites which are removed by β -galactosidase treatment, over and above the removal by neuraminidase treatment (Fig. 2).

In differentiated cultures 54 % was bound and 46 % displaced by the native unlabelled toxin. As we have shown above, 52 % of the total binding was sialic-acid dependent. If we assume that the non-displaceable association would be of the same nature in both types of culture, then differentiated cells have about 55 % of class 1(b). The non-displaceable corresponds roughly to the amount attributed to sialic-acid dependent binding. The remaining 45 % is accounted for by β -galactoside-linkage dependent sites and the independent ones, the sum of which totals above to 48 % (± 3.1) of the total binding.

The $t_{1/2}$ ON for differentiated cell specific binding = 10 sec. The calculated $k_1 = 6.93 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$, and represents $1.361 \times 10^{-4} \mu\text{g}$ toxin per cell population or $5.79 \times 10^{-7} \mu\text{g}$ toxin per μg cell protein.

Dissociation Growth Cultures

A. Native Toxin

After pre-incubation of growth culture cells with $\{^{125}\text{I}\}$ -toxin for 30 min at 4°C native toxin was added. 50 % of the specific binding had been removed after 15 sec ($t_{1/2}$ OFF) and the calculated $k_1 = 4.62 \times 10^{-2} \text{sec}^{-1}$. $K_D = k_{-1}/k_1 = 3.473 \times 10^{-13} \text{M}$ (Fig. 3a).

B. Neuraminidase and β -galactosidase

100 % of the cpm bound was removed by application of either enzyme. Kinetics here would be attesting to the specific activity of the enzymes only in presence of a bound substrate. Although the toxin was bound to the sialic-acid containing residues, it did not block the action of the enzymes. (Fig. 3b and c).

Dissociation of Differentiated Cultures

A. Native Toxin

The specifically bound $\{^{125}\text{I}\}$ -toxin was rapidly dissociated from the differentiated cells, $t_{1/2}$ OFF = 1 min. The calculated $k_{-1} = 3.30 \times 10^{-3}$. $K_D = 4.761 \times 10^{-14} \text{M}$ (Fig. 3a).

B. Neuraminidase and β -galactosidase

Differentiated cells show an unusual pattern for enzymatic dissociation. After 15 min the maximum is removed by either enzyme then cpm rise again. There was no free toxin in the medium at the beginning of the experiment, thus the toxin removed by neuraminidase action ($36.5 \% \pm 2.1$) was all rebound within an hour, giving a V-shaped curve (Fig. 3b). Similar results were obtained using β -galactosidase. A total of $62.5 \% (\pm 1.7)$ of the bound toxin was removed in 15 min. The re-binding rises to 60 % of the original cpm bound (Fig. 3c).

Reverse Displacement

Differentiated cells having been pre-incubated with unlabelled toxin for 30 min at 4°C were handled in the following manner :

- Group a) exposed directly after washing to $\{^{125}\text{I}\}$ -toxin
- Group b) exposed 30 min to 0.1 % neuraminidase
- Group c) exposed 30 min to 0.1 % β -galactosidase
- Groups b) and c) were then exposed to the iodinated toxin.

Control cultures were run for total $\{^{125}\text{I}\}$ -toxin binding

Pre-incubated native toxin directly displaceable by the labelled toxin amounted to 11.5 % (± 2.3) of the total control (^{125}I)-toxin binding (Fig. 5) which would be thereby 25 % of the "specific" binding corresponding to that which was displaced in the (^{125}I)-toxin by native reverse operation (Fig. 4).

Treatment with neuraminidase released an additional 37 % (± 2.4) of total control sites, giving a total of 48.5 % of the gross binding. This percentage correlates well with the non-displaceable Class 1b demonstrated in figure 1.

After β -galactosidase treatment an additional 47 % (± 1.7) of sites were liberated and 96.5 % (± 2.7) of the control gross total was achieved. It would appear that more native toxin was therefore bound to β -galactoside-dependent linkage than in the case of the iodinated analog, where only 22 % of the total sites were released by β -galactosidase but not by neuraminidase. This percentage, however, constitutes about 50 % of the specific (displaceable) binding for (^{125}I)-toxin (Fig. 1) which is in fairly close agreement with the native toxin figure (47 %). In these experiments there is no way to estimate the actual amount of native toxin which was bound. Other tests are planned to clarify this matter.

Growth cultures showed no reverse displacement at all in these assays.

Binding Assays in other Cell Cultures

A maximum of 89 cpm was achieved in the three culture types tested : monkey kidney CV1, calf lung and chicken erythrocytes. This amount was not reproducible, and therefore is considered insignificant. There was no increase, regardless of temperature and amount of time of incubation (Fig. 1a).

Displacement of (^{125}I)-toxin with Toxoid

Tetanus toxoid used in large excess did not displace any binding of (^{125}I)-toxin previously bound to cells. Furthermore, there was no change in association in either type of cell culture. (Data not shown). Since (^{125}I)-toxoid did not bind under our experimental conditions, these results were as expected.

Binding of Different Concentrations of (^{125}I)-Toxin

Additions of 10^{-10} to 10^{-16} moles of (^{125}I)-toxin were assayed for binding. Due to the diluting, the two minimal concentrations were all fixed on the cell membranes and it was not until the 10^{-14} concentration of moles that there was FREE unbound toxin. From that point on, no matter how many cpm were applied, the same mean cpm were bound. Had a toxin of higher specific activity been used, it is probable that there would be further reduction in the biological activity since the present labelling was only about 80 % efficient (Fig. 5).

DISCUSSION

We have demonstrated three classes of (^{125}I)-toxin binding sites on the surface of neuroblastoma cells in these experiments ; Class 1, sialic acid residue dependent, Class 2, β -galactoside linkage dependent, and Class 3, independent.

Only Class 1 is shown in growth cultures, which is both neuraminidase and β -galactosidase sensitive. Due to this result, we assume that since the sialic acid dependent binding sites were also removed by clearance of β -galactoside base,

thus the sialic acid residues were concomitantly removed. The shape of the curve indicates that there might be a possibility of partial reliance upon the terminal galactose as well, and that with the course of the enzyme action the terminal 1-3 β -galactoside linkage is cut first, then the linkage farther within the ganglioside residue "behind" the component sialic acids (fig. 3b).

Using visual morphological criteria, the binding to growth cultures has been previously described as **INEFFECTIVE**, since there was no change in viability or division of the cell when exposed to even very high concentrations of tetanus toxin (Zimmerman and Piffaretti, 1977). Due to the fact that tetanus toxin has not been shown to bind to cells other than those with neuronal characteristics, the fixation is all, certainly, tissue specific. A large part of the binding demonstrated in these experiments could not be displaced with an excess of native toxin. However, since 47 % of the sites bound are reproducibly displaceable this separates Class 1 (sialic acid residue dependent) receptors into two groups : one (a) which is displaceable and by classic definition "specific", and another (b) non-displaceable and thereby "non-specific" (Cuatrecasas and Hollenberg 1976). Both groups could be associated with gangliosides, possibly the di- and trisialogangliosides demonstrated by van Heyningen (1974) to bind tetanus toxin.

For each type of ganglioside receptor on the growth cell surface we propose a separate site on the toxin molecule situated such that binding to one excludes the binding of the other. The actual fixation to a given receptor is therefore a function of the concentration and dispersion of the particular discriminator on the membrane, the probability of "hit" by the appropriate determinant on the toxin molecule, and the relative affinity for the receptors in question. The net result of this is that at equilibrium 53 % of the (^{125}I)-toxin was bound to Class 1b (non-displaceable) sites and 47 % was bound to Class 1a (displaceable) receptors. During the period of time to establish the equilibrium transition phases arrived such that relatively more toxin was bound to one site than the other. This is no doubt due to the dynamic situation of binding and release of attachment at any given site and the renewal of the original probability of re-fixation thereto or to the other site. Since these binding studies were carried out at 4° C we have practically eliminated the interference of the pinocytosis demonstrated in our fluorescent studies, therefore the binding measured is just that and does not involve the complication of internalization of the toxin.

Differentiated Cells

Linked with our previously demonstrated **EFFECTIVE** binding in morphologically differentiated cells (Zimmerman and Piffaretti, 1977) is a considerably more complex cell surface receptor spectrum. Differentiated cells show three classes of binding sites : Class 1, neuraminidase sensitive, Class 2, β -galactoside linkage dependent, and Class 3, independent of either. The displaceable binding amounts to 46 % of the total binding at equilibrium. This coincides closely with the figure for growth culture cells. Due to the **EFFECTIVE** nature of the binding in differentiated cultures, it is reasonable to assume that the non-displaceable-site-bound toxin is the same in both types of culture, and it is the displaceable which is different. The β -galactoside linkage dependent class plus the independent class total to 46 % of the total binding. We conclude at least provisionally that these are the **EFFECTIVE** sites, and that the ganglioside, Class 1 b sites common

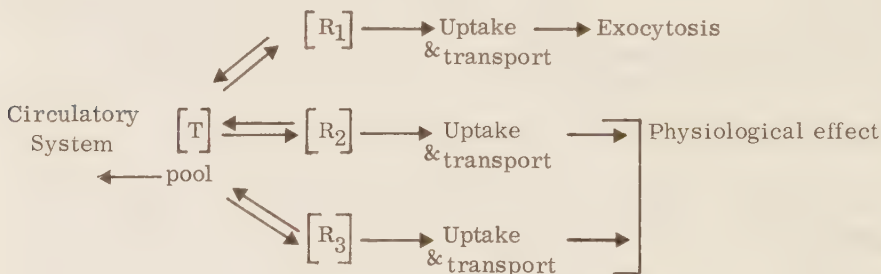
to both are INEFFECTIVE as well as are the Class 1a found in growth cells. This leaves Classes 2 and 3 as EFFECTIVE binding, both displaceable by the native unlabelled toxin. In vivo retrograde transport studies (Stöckel et al 1977) have shown that pre-incubation with gangliosides inhibited transport a maximum of about 40 %. These results correlate well with our distribution of classes of sites in the binding studies. We feel that the ganglioside bound toxin is taken up, but with an INEFFECTIVE result on the cell metabolism or morphology. On the other hand, it is likely that the displaceable binding, also internalized, has secondary binding within the cell thus rendering it EFFECTIVE. Further studies are needed to elucidate this. Kryzhanovski (1975) demonstrated toxin fixation to the mitochondrial fraction of disrupted nervous tissue. Also of interest, is that in the same studies toxoid was also bound on the same sub-cellular fraction. These results support our hypothesis that the toxoidization renders the toxin incapable of binding to the surface membrane but does not detoxify it as such.

Gangliosides have been for several years accepted as the specific receptor for tetanus toxin, to the extent that not much research has been carried out looking for something else. With the development of this tissue culture system using mouse neuroblastoma cells we have been able to demonstrate and partially characterize other receptors. Although the argument may be raised that these are tumour cells, we feel that the information obtained about the binding affinity for different classes of sites provides a spring-board for further nerve membrane studies. The high values for the kinetics constants correlate well with the molarities found in vivo for the minimum lethal dose and indicate, on the molecular level the critical amounts to achieve the biological effect.

In the case of tetanus toxin, we are not dealing with a simple relationship.



We propose a complex network of model interaction :



Schwab and Thoenen (1976) presented evidence for transsynaptic migration of tetanus toxin. Bearing in mind the above, plus our results and the derived model, consider the possibility that the $[R_2]$ and $[R_3]$ bound toxin is taken up, transported and remains in the first cell body and binds internally, resulting in a physiological effect in that cell. The $[R_1]$ bound toxin could be that, therefore which is also internalized and transported, but is also released at the next synapse and faces a new network of available receptors on the next cell. The nerve to nerve dissemination of tetanus would, in this model, be more dependent on the non-displaceable $[R_1]$ binding. The local effect, therefore, is dependent on $[R_2]$ and $[R_3]$ bound toxin. The ultimate physiological effect will be found when a relationship can be established between the Class of receptor to which the toxin is bound, its fate within the cytoplasm, and a measurable change in the functional metabolism of the cell.

We have shown a biological activity for tetanus toxin in tissue culture. Further studies are proposed to correlate this reversal of morphological differentiation with physiochemical criteria of differentiated functions, i.e. the release of neurotransmitters. Separate experiments have already been carried out to begin to investigate the nature of the reversal of cell processes (manuscript in preparation). In this direction we are trying to orient our future research toward better understanding of the mechanism of the disease.

Acknowledgements. We are indebted to Professor H. Thoenen and Dr M. Schwab for fruitful discussions and advice in the preparation of this manuscript. We are grateful for the generous gift of the neuroblastoma cells by Dr D. Monard, and of the tetanus toxin by Dr B. Bizzini. We thank Miss Colette Pasquier and Miss Brigitte Friedli for their excellent technical assistance. We appreciate the valuable assistance of Mrs. Virginia Ising-Hose in the preparation of this manuscript.

TABLE I

Characteristics of (125 I) Tetanus Toxin Binding

Differentiated Cells

Binding	CPM	μ g Toxin	Moles	Molecules
Total	5200	2.954×10^{-4}	$1.969 \times 10^{-15} \text{M}$	1.86×10^9 Molec $1.702 \times 10^3/\text{cell}$
Specific	2392	1.361×10^{-4}	$0.907 \times 10^{-15} \text{M}$	5.46×10^8 Molec $7.84 \times 10^2/\text{cell}$
NA ^{ase} Independent	2494	1.418×10^{-4}	$0.945 \times 10^{-15} \text{M}$	5.69×10^8 Molec $8.17 \times 10^2/\text{cell}$
NA ^{ase} Dependent	2756	1.565×10^{-4}	$1.04 \times 10^{-15} \text{M}$	6.26×10^8 Molec $8.99 \times 10^2/\text{cell}$
β -Gal-ase Dependent	1144	0.65×10^{-4}	$0.43 \times 10^{-15} \text{M}$	2.60×10^8 Molec $3.74 \times 10^2/\text{cell}$
Independent	1352	0.768×10^{-4}	$0.51 \times 10^{-15} \text{M}$	3.08×10^8 Molec $4.42 \times 10^2/\text{cell}$
Growth				
Total	3296	1.872×10^{-4}	$1.248 \times 10^{-15} \text{M}$	7.519×10^8 Molec $1.60 \times 10^3/\text{cell}$
Specific	1549.12	0.88×10^{-4}	$0.58 \times 10^{-15} \text{M}$	3.53×10^8 Molec $7.53 \times 10^2/\text{cell}$

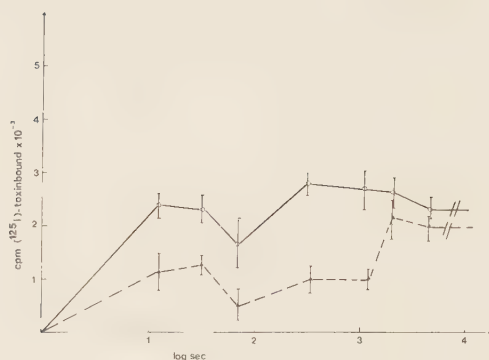
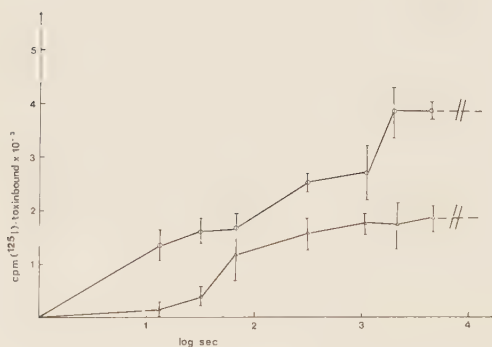
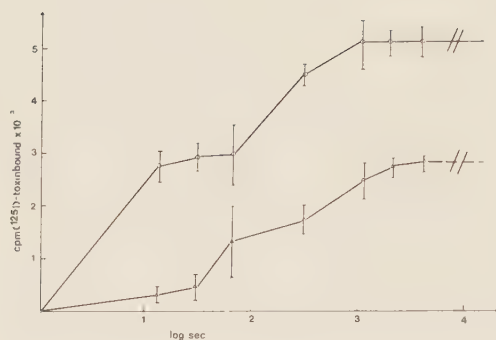
TABLE II

Kinetics Constants of Tetanus Toxin Binding
To Neuroblastoma Cells

Culture Binding	k_1	k_{-1}	KD
Total	$3.00 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$	-	-
Growth			
Specific	$1.33 \times 10^{11} \text{M}^{-1} \text{s}^{-1}$	$4.62 \times 10^{-2} \text{sec}^{-1}$	$3.47 \times 10^{-13} \text{M}$
Total	$5.33 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$	-	-
Differentiated			
Specific	$6.93 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$	$3.30 \times 10^{-3} \text{sec}^{-1}$	$4.76 \times 10^{-14} \text{M}$
Nase Indep.	$2.75 \times 10^9 \text{M}^{-1} \text{s}^{-1}$	-	-
Indep.	$1.68 \times 10^9 \text{M}^{-1} \text{s}^{-1}$	-	-
β -galac- toside depen- dent	-	-	-

Fig. 1

Time-course of the association of (125 I)-tetanus toxin with mouse neuroblastoma cells in a) growth culture cells, O—O total binding; Δ — Δ in presence of unlabelled native toxin, b) differentiated cells, O—O total binding; Δ — Δ in presence of unlabelled native toxin, c) displaceable binding in $\blacktriangle$ — $\blacktriangle$ growth and O—O differentiated cell cultures. Each point represents the mean $\pm$ S. E. M. (n=12).



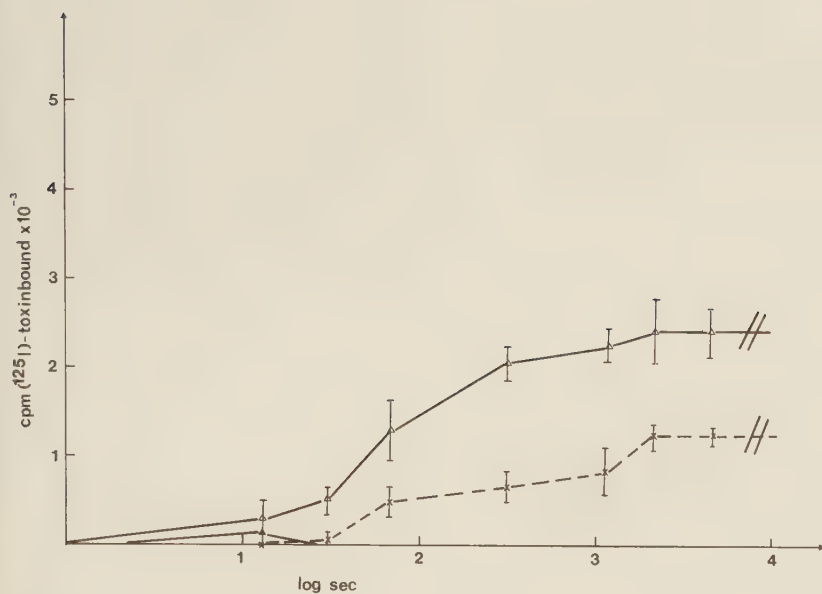
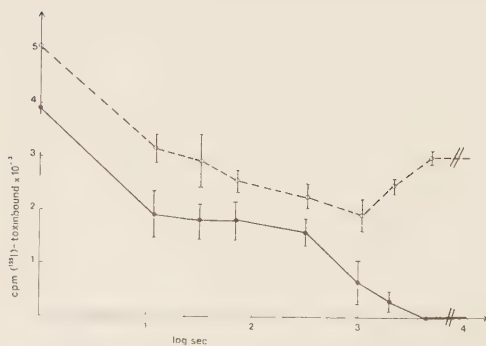
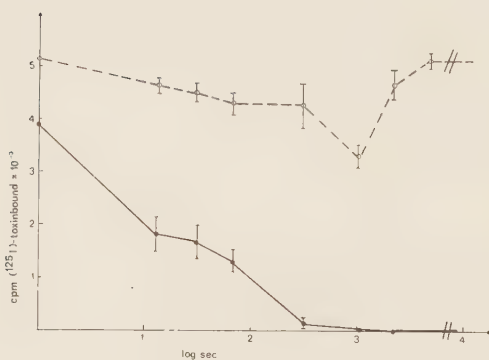
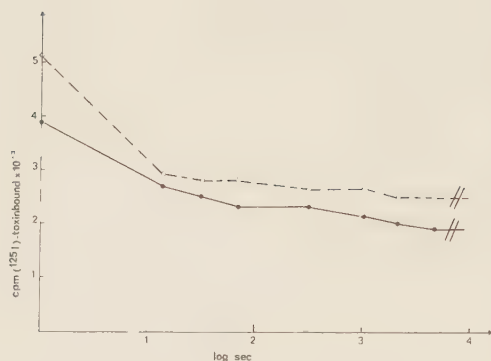


Fig. 2 : Time-course of the association of (^{125}I) -tetanus toxin in presence of enzymes : $\blacktriangle$ — $\blacktriangle$ growth cells in presence of either 0.1 % neuraminidase or 0.1 % β -galactosidase ; $\triangle$ — $\triangle$ differentiated cells in presence of 0.1 % neuraminidase ; X—X differentiated cells in presence of 0.1 % β -galactosidase. Each point represents the mean $\pm$ S. E. M. (n=10)

Fig. 3 :

Dissociation of bound (^{125}I)-tetanus toxin : a) by native unlabelled toxin in $\bullet\text{---}\bullet$ growth cultures and O---O in differentiated cultures. b) by 0.1 % neuraminidase action in $\bullet\text{---}\bullet$ growth cultures and O---O in differentiated cell cultures. c) by 0.1 % β -galactosidase action in $\bullet\text{---}\bullet$ growth cultures and O---O in differentiated cell cultures. Each point represents the mean $\pm$ S. E. M. (n=12)



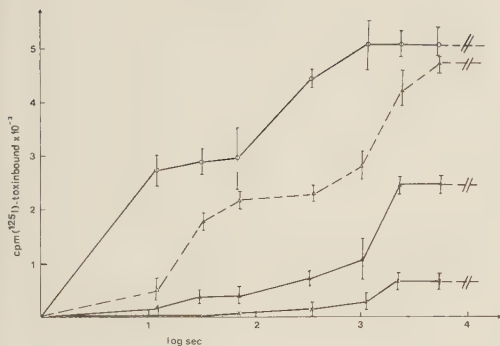


Fig. 4 :

Displacement of bound native unlabelled toxin by (^{125}I) -toxin in differentiated cell cultures by X—X. (^{125}I) -toxin added directly after pre-incubation ;
 ▲—▲ exposure to (^{125}I) -toxin after 30 min treatment with 0.1 % neuraminidase or Δ Δ 0.1 % β -galactosidase ;
 O—O is the control without pre-incubation with native toxin.
 Each point represents the mean $\pm$ S. E. M. (N=10)

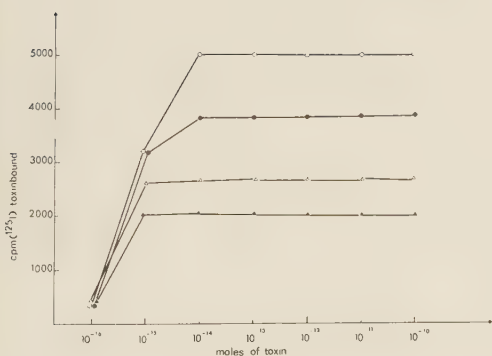


Fig. 5 :

Association of 10^{-10} to 10^{-16} moles of (^{125}I) -tetanus toxin in ●—● growth and O—O differentiated cultures. ◆ = total cpm of that concentration, therefore, no free unbound toxin. This plot represents total binding at equilibrium (60 min) and in separate tubes incubated for 2 h the binding did not increase. The lower curves represent binding of (^{125}I) -toxin in presence of an excess of unlabelled native toxin ▲—▲ in growth cells and Δ—Δ in differentiated cultures. The non-displaceable binding is maximum at 10^{-15} M.

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PART III

INTERACTION OF TETANUS TOXIN WITH CULTURED NEUROBLASTOMA CELLS : A BIOLOGICAL ACTIVITY OF TETANUS TOXIN IN VITRO

SUMMARY

We have previously demonstrated by using immunofluorescence and (^{125}I)-labelled toxin, that the tetanus toxin molecule binds selectively to neuroblastoma cells in tissue culture. Three classes of cell surface receptors have been characterized in morphologically differentiated cells, induced to form neurites by depriving them of serum (Zimmerman and Piffaretti, 1977a, 1977b). These experiments show that the toxin also binds to differentiated cells stimulated by other inducers of neurite extension ; glial cell conditioned medium (GCCM), aminopterin, and dibutyryl 3', 5' adenosine monophosphate. Only in the glial cell conditioned medium, and the serum free DMEM induced cells was the morphological differentiation reversed, thus EFFECTIVE binding. With the other stimulators there was no observed change in the formed processes. We suggest that there is therefore a difference in the membrane constituents dependent upon the manner of induction of neurite extension.

KEY WORDS : Differentiation - neuroblastoma cells - tetanus toxin - inducers - neurites.

RUNNING TITLE : Tetanus Toxin reverses neurite extension.

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INTRODUCTION

With our primary observations of neuroblastoma cells (clone NB2A) exposed to tetanus toxin in DMEM without serum, we noted the reversal of morphological differentiation (Zimmerman and Piffaretti 1977a). In these experiments, we have utilized other methods of process induction to determine further the nature and scope of this biological activity. Tests were run on known modes of induction of neurite extension and their sensitivities to inhibitors, colchicine, cytochalasin B and tetanus toxin. Evidence presented here indicates that the methods can be separated primarily into two classes on the basis of the ability of the two reagents (Furmanski, 1973), and secondly, on the sensitivity to tetanus toxin to cause retraction of the neurites formed. Cells incubated in the presence of serum and either dibutyryl cyclic-AMP or aminopterin were insensitive to tetanus toxin. Additions of toxin to cells in serum-free DMEM or

glial cell conditioned medium resulted in reversal of morphological differentiation. No correlative assays were conducted on the biochemical criteria for further definition of the differentiated cells.

Our results with glial cell conditioned medium and serum-free DMEM show that high concentrations of toxin yield a dynamic retraction of processes within 4 h. The neurites appear to reverse from long to short processes at one level of sensitivity, but the total disappearance in GCCM does not occur regardless of concentration used. Since in serum-free DMEM the critical concentration is much lower, it positively demonstrates the effect of the active substance in the GCCM (Monard et al 1973).

Using lower concentrations of toxin, it took 24 h to achieve similar results. This suggests that the critical amount to effect the reversal accumulates in 4 h at high concentrations and takes 24 h to accumulate at low concentrations. It also casts doubt on the possibility of membrane modifications due only to the toxin binding as the cause of the reversal.

Binding studies with (^{125}I)-toxin showed that the binding occurs in each of the induced cultures. Sensitivities to neuraminidase and β -galactosidase vary with the EFFECTIVE and INEFFECTIVE binding.

MATERIAL

Tetanus Toxin was a gift of Dr B. Bizzini, Institut Pasteur, Paris.

Mouse Neuroblastoma Cells C1300 (clone NB2A) were gift of Dr D. Monard, Friedrich Miescher Institut, Basel.

Dulbecco's Modified Eagle's Medium (DMEM) was obtained from GIBCO.

Fetal Calf Serum (FCS) was from Colorado Serum Co.

Plastic Petri Dishes from Falcon.

12 x 32 mm Glass Coverslips were from Auer, Bittmann and Soulier, Basel.

Theophylline and Dibutyryl Cyclic Adenosine Monophosphate (Bt₂cAMP) were purchased from SIGMA.

Aminopterin was from SERVA.

Glial Cell Conditioned Medium, prepared as described previously (Monard et al 1973) was obtained from Dr D. Monard.

Carrier free (^{125}I) Na was purchased from the Radiochemical Center, Amersham, UK, Specific activity 8-14 Ci/mg Iodine.

METHODS

Assays were conducted to characterize the inducers and inhibitors of morphological differentiation in clone NB2A neuroblastoma cells. The inducers used were serum free DMEM, glial cell conditioned medium (Monard et al 1973) serum free conditioned medium, $5 \times 10^{-7}\text{M}$ aminopterin and 1mM Bt₂cAMP

(Furmanski et al 1971). 5×10^4 cells were seeded in 60 mm diameter plastic petri dishes containing three glass coverslips each. After 24 h growth at 37° in 5 % CO_2 atmosphere in DMEM + 10 % FCS, the medium was removed and changed as appropriate. Chemical inducers were added to fresh DMEM + 10 % FCS. Cells were incubated for 48 h until neurites were well formed, observed and processes counted.

Morphological differentiation was recorded as : the total number of cells with processes of > 1 cell diameter in length, between 1 and 2 cell diameters in length, and those which were > 2 cell diameters long. Percentage of cells with each category of neurite represents the absolute percentage, and not a percent of control.

Two methods of checking the effects of colchicine, cytochalasin B and tetanus toxin upon axon formation were carried out : a) adding inhibitor at the same time as the inducer and b) adding the inhibitor after the processes had been induced to observe reversal of morphological differentiation. Colchicine was used routinely at $1 \mu\text{g/ml}$ and cytochalasin B at $2 \mu\text{g/ml}$ (Furmanski, 1973). Tetanus toxin was added to a final concentration of $12.7 \mu\text{g/ml}$ for all tests. Cytochalasin and cytochalasin B have been shown to inhibit neurite outgrowth at concentrations of 10^{-7} and 10^{-6}M respectively (Seeds, et al, 1970). Since cyclohexamide was found to have little effect at concentrations up to $1.8 \times 10^{-4}\text{M}$, it was not tried in these experiments.

For other toxic effect evaluation experiments, 5×10^4 mouse neuroblastoma cells were inoculated into 60 mm $\emptyset$ plastic petri dishes containing three glass coverslips and grown in DMEM + 10 % FCS. All serum used in these experiments was inactivated by heating to 55° for 30 min. Cultures were incubated at 37°C in a 5 % CO_2 + 95 % air atmosphere. After 24 h the medium was siphoned off and glial cell conditioned medium was added to induce process formation. Incubation was continued for 48 hours until processes were well formed (Monard et al 1973). Processes were observed and counted. 500 cells were counted in randomly selected microscope fields. The areas were circled on the bottom of the petri dish for later counts, so that the same cells were observed at the different time intervals. Dishes were separated into four sets for toxin assays and fresh media were added : a) DMEM + 10 % FCS ; b) serum-free DMEM ; c) conditioned medium, and d) serum-free conditioned medium. Each set contained five plates, one for each concentration of toxin. In test I, dilutions of toxin containing final concentration of $1.6 \mu\text{g/ml}$, $4.7 \mu\text{g/ml}$, $14.2 \mu\text{g/ml}$, $42.6 \mu\text{g/ml}$ and $127.8 \mu\text{g/ml}$ were added accordingly. Controls for each medium were conducted in parallel. After 4 h the cells were observed and counted as above.

In a second test, more diluted toxin was used to attempt to determine the minimum concentration to yield a reversal of processes. Toxin was added ; $1.6 \mu\text{g/ml}$, $0.8 \mu\text{g/ml}$, $8 \times 10^{-3} \mu\text{g/ml}$, $8 \times 10^{-5} \mu\text{g/ml}$ (concentration as in (^{125}I) -toxin binding experiments). The same procedures described above were used in these experiments.

Cell protein was determined by the technique of Oyama and Eagle (1956), a modification of the method of Lowry et al (1951).

Tetanus toxin was also pre-incubated with each of the inducers to determine whether the reversal was an inactivation effect or directly related to the cell.

Tetanus toxin was labelled with (^{125}I) Na as previously described (Zimmerman and Piffaretti, 1977). The specific activity of the iodinated protein was $7.95 \mu\text{Ci}/\mu\text{g}$ toxin. The final concentration of toxin was about $185 \mu\text{g}$ protein per ml. Protein content was determined by the method of Lowry et al (1951).

Binding assays were conducted for each of the inducers : conditioned medium, serum free conditioned medium, Bt_2 cAMP, aminopterin, serum free DMEM, after 48 h of differentiation, $10 \mu\text{l}$ of diluted labelled toxin (3.3×10^5 cpm) were added to each dish and maintained at 4°C . Recordings up to one minute were done separately and individually, with readings at 15 sec, 30 sec and one min. Toxin was added during gentle rotation of the dish, coverslips were removed, washed three times in three changes of 0.15M PBS (pH7.4) and placed directly into counting tubes. Control blanks were run during each phase of the experiment.

Exposure to (^{125}I)-toxin was continued and sampled during 3 h. All dissociation experiments were done with an excess of native toxin after 60 min pre-incubation with (^{125}I)-toxin at 4°C . This probably avoids the complication of internalization of the toxin.

Separate tests were carried out to evaluate the effect of elevated levels of cAMP on the morphological differentiation. Theophylline (Furmanski et al, 1971) was added to the culture medium to a final concentration of 10^{-7}M , with and without tetanus toxin ($12.7 \mu\text{g}/\text{ml}$). Trials were done in serum free DMEM and in conditioned medium with and without FCS. Cells were observed and counted as described above.

RESULTS

Observations of Neurite Extension :

Where either the serum is removed, or other morphological differentiation inducers are added, the cells flatten out and axons are extended. Clone NB2A of neuroblastoma is constituted with several different forms of neuronal cell ; those which are of average size (approximately 20μ in diameter) which extend two polar processes, large cells of about 50μ in diameter which extend 5-7 neurites which branch, and kite shaped cells which form three axons. All forms are visible regardless of the manner of stimulation. With serum-free DMEM, glial cell conditioned medium, serum-free conditioned medium and Bt_2 cAMP, the cells look pretty much alike. After 48 h, neurites are long and interlaced into networks. The populations are about equal in number, whereas with aminopterin, the cell count is greatly reduced and the axons are 2-3 times as long. Other experiments in which we used dimethyl sulfoxide (DMSO), there was a lag period of up to 48 h before neurites appeared and then they were much thicker than those found by other methods (unpublished observations).

In our routine counts, less than 1 % of cells had no process and the rest were divided into the three classes, > 1 cell diameter, between 1 and 2 diameters, and > 2 cell diameters in length.

Inhibitors of Neurite Outgrowth :

Results of assays either by co-incubation of inhibitors with cells or additions of inhibitor after pre-formation of neurites are shown in Table I. Aminopterin and Bt_2 cAMP formed neurites were insensitive to tetanus toxin.

Tetanus Toxin Reversals of Morphological Differentiation :

Cells were induced to extend neurites by 48 h exposure to glial cell conditioned medium. The medium was siphoned off and replaced by : DMEM + 10 % FCS, serum-free DMEM, conditioned medium or serum-free conditioned medium. Tetanus toxin was added to each set and cells were observed after 4 h. (Fig. 1). The concentration for 50 % reversal of morphological differentiation at 4 h (RMD₅₀₄) was calculated for total processes (≥ 1 cell diameter) and for long processes (> 2 cell diameters in length) for each medium. Lower concentrations of toxin were used for comparison which were in the range of the protein content of the (¹²⁵I)-labelled toxin used in binding experiments. These cells were observed during 24 h at which time the effects were comparable to the 4 h results obtained with the higher concentrations (Fig. 2), giving RMD₅₀₂₄.

The effect of $10^{-7}M$ theophylline was measured on cells in DMEM + 10 % FCS, serum-free DMEM, glial conditioned medium, and serum-free conditioned medium having been induced to morphological differentiation with glial conditioned medium (Fig. 3). The processes formed were doubly tested by the addition of tetanus toxin. The theophylline inhibition of phosphodiesterase appeared to protect total retraction of the process in presence of serum, but the long (> 2 cell diameter) processes were retracted almost entirely after 4 h. In absence of serum, in the conditioned medium, we noted less reversal in presence of theophylline. The > 2 diameter processes were much longer and with addition of toxin, 40 % neurites still remained. There is a net difference in this effect in routine DMEM (not conditioned by glial cells).

¹²⁵I-Tetanus Toxin Binding to Cells Induced to Differentiate :

Neuroblastoma cells stimulated to extend neurites by the various experimental means all bound (¹²⁵I)-tetanus toxin (Fig. 4). The half saturation time was longer than in cells induced by depriving them of FCS. Binding in presence of an excess of non-iodinated native toxin showed that an average of 47 % of the total binding was displaceable, which is similar to the percentages obtained previously. (Zimmerman and Piffaretti, 1977b). Association in presence of 0.1 % neuraminidase or β -galactosidase confirmed earlier data about EFFECTIVE binding (Fig. 4c).

Dissociation of pre-saturated (¹²⁵I)-toxin with native toxin was rapid at first, then leveled out. Half of the bound displaceable toxin was removed in 5 min in conditioned medium cultures and in 7 min in Bt_2 cAMP or aminopterin culture. Kinetics constants are given in Table II.

Removal of the bound labelled toxin by enzyme action followed the expected pattern for EFFECTIVE and INEFFECTIVE binding (Fig. 5).

Table III shows a comparison of the binding capacities of cells induced by the different culture conditions.

DISCUSSION

Neurites extended by neuroblastoma C1300 (clone NB2A) cells in culture are retracted in presence of colchicine, cytochalasin B and tetanus toxin. The sensitivity of the formed processes is dependent upon the method used for induction of differentiation.

Our results with tetanus toxin reversal of morphological differentiation indicate the possibility of two stages in process extension. There appears to be a difference in the retractability of long (greater than 2 cell diameters in length) processes and shorter ones, the length of which is about equal to the diameter of the cell. In DMEM + 10 % FCS, the total number of neurites descended gradually with the increase of toxin concentration until at 48.2 $\mu\text{g/ml}$ 50 % were reversed, while the long processes retracted 50 % in presence of the minimum toxin addition in the high concentration experiment. In the absence of FCS, the 50 % level was reached at the second concentration (4.7 $\mu\text{g/ml}$). At a concentration of 18.2 $\mu\text{g/ml}$ in the same experiment 50 % of total processes were retracted. The serum in this case seems to "protect" the short processes.

The pattern is quite different with cells maintained in glial cell conditioned medium. The long processes in presence of serum are maintained until, at a concentration of 17.8 $\mu\text{g/ml}$ they abruptly are reversed and at 42.6 $\mu\text{g/ml}$ have disappeared completely. On the other hand, the short processes are only 50 % retracted at 127.7 $\mu\text{g/ml}$. In absence of serum, the long processes are 50 % reduced at 9.4 μg toxin per ml, but the short ones were maintained after retraction from the greater length at 50 % of the control no matter what concentration was used. In either case, one can see the step-wise dynamics of retraction before the longer processes are completely eliminated (Fig. 1).

Experiments were also conducted with weaker concentrations of toxin. After 24 h, control cultures showed a predominance of longer processes except in DMEM + 10 % FCS where the cells were all round. Partial reversal was achieved at the lowest concentration used (8×10^{-5} $\mu\text{g/ml}$) in each type of culture. 50 % of the cells had no processes at all in serum free DMEM or glial cell conditioned medium at 8×10^{-1} μg toxin per ml. At 1.6 $\mu\text{g/ml}$, the processes in serum free conditioned medium were reduced 50 %. From these results we conclude that even at very feeble concentrations the binding is EFFECTIVE in these cultures.

In the (^{125}I)-toxin binding assays we have noticed a difference in the character of the receptors depending upon the mode of induction of the cells to differentiate. The binding is INEFFECTIVE in both Bt_2cAMP and aminopterin induced cells (i.e. no process retraction). The mechanism of the action of tetanus toxin seems to be related to neither colchicine nor cytochalasin B. Bt_2cAMP induced processes are sensitive to cytochalasin B and only partly sensitive to colchicine,

whereas the aminopterin stimulated neurites are resistant to cytochalasin B and sensitive to colchicine. Serum free DMEM induced cells are sensitive to all three.

As it has been shown previously in growth culture cells (Zimmerman and Piffaretti 1977), the binding is sensitive to neuraminidase and β -galactosidase. Whereas in growth cultures, the total binding is removed by neuraminidase or β -galactosidase action and there is no association in presence of either enzyme, in Bt₂cAMP and aminopterin induced cells there is a difference between the action of the two enzymes. Neuraminidase removed all but about 15 % of the bound (¹²⁵I)-toxin, and β -galactosidase removed all of it. We interpret this as the presence of two classes of INEFFECTIVE binding, one which is dependent upon a neuraminidase-sensitive residue and another which requires a β -galactoside linkage. These could both well be on the ganglioside residue, however, and some fixation depends upon, say, the terminal galactose.

The same classes of receptors appear in glial cell conditioned medium induced cells as do in the other EFFECTIVE binding for serum free DMEM cultures. About 53 % of the sites are neuraminidase sensitive (Class 1), 26 % are (β -galactoside linkage dependent (Class 2) and 20 % are independent (Class 3).

About 50 % of the binding is displaceable in all induced cultures. Considering that Stöckel et al (1977) have shown that tetanus toxin is still transported intra-axonally after pre-absorption with gangliosides, but to a lesser extent, we have presumed all binding to be tissue specific. The displaceability therefore relates to the destiny of the toxin vis-a-vis pathogenic site for activity within the cell and not to the "specificity" per se.

CONCLUSIONS

From the results in these experiments, we have concluded that the cell surface receptors for tetanus toxin in morphologically differentiated cells differ, depending upon the method of induction. The binding in Bt₂cAMP and aminopterin induced cells is INEFFECTIVE. The mechanism of tetanus toxin reversal of morphological differentiation is different from either colchicine or cytochalasin B. There appear to be two levels of sensitivity to tetanus toxin in the formed neurites. The long processes are more easily reduced, but the shorter ones show greater tolerance for the tetanus toxin action. This difference could be accounted for by two messages or decisions on the part of the cell : one, to form a process and two, to extend it.

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GENERAL CONCLUSIONS

Although there has been extensive research devoted to Tetanus for nearly a century, the actual mechanism of the pathogenesis still remains a mystery. The protein exo-toxin has been purified by several laboratories and characterized as to amino acid content, molecular weight and iso-electric point. The overall molecular structure is still a matter of conjecture, however. It has been shown that the toxin is transported intra-axonally in vivo by several laboratories. The "bridge" between the isolation and study of the toxin and the transport internal to the axon lies at the action of the toxin at the membrane level, the cell surface receptors.

The work presented here demonstrates a research system, actually "mini-in-vivo", since in tissue culture mouse neuroblastoma cells behave like neurons in that they produce neurotransmitters, differentiate morphologically and respond to electric stimuli. These characteristics make them an ideal "laboratory animal" to test a disease of the nervous system.

The toxin binds to the intact cell, the toxoid does not. These results suggest that the process of toxoidization damages the binding unit on the molecule.

In growth cultures the binding is INEFFECTIVE, in that there is no visible change in cell comportment or division in presence of toxin. This binding is dependent upon sialic-acid residues and can be removed entirely using neuraminidase or β -galactosidase treatments. The binding is also INEFFECTIVE in cells induced to differentiate by the addition of aminopterin or dibutyryl-cyclic AMP. Bound toxin is likewise removed by the action of the two enzymes.

Cells induced to differentiate by removal of fetal calf serum or addition of glial cell conditioned medium respond by retraction of formed neurites. This binding is termed EFFECTIVE. The reversal of morphological differentiation of the toxin is parallel to neither that of colchicine nor cytochalasin B. We conclude thereby that the action apparently is not directly related to either microtubule or microfilament inhibition. Since cyclohexamide, which reacts with the 80S ribosome does not reverse the processes, and tetanus toxin does, we propose that there might be inter-action of tetanus toxin with the mitochondrial fraction of cells. Kryzhanovski has previously found binding of labelled toxin and toxoid to this sub-cellular fraction.

We have shown in these experiments two supplementary classes of receptors linked to the biologically EFFECTIVE binding: β -galactoside linkage dependent and that which is independent from the sites of the action of either enzyme used. Further research is needed to isolate and characterize the relationship of these sites to the ultimate physiological effect.

We have demonstrated a high kinetics constant ($K_D = 10^{-14}M$) which correlates well with other studies reporting a calculated $1/LD_{50}$ equal to 10^{-15} Molar.

The kinetics of the binding, together with the morphological discernable biological activity give a spring-board for further research toward the elucidation of the physiological activity, and from there toward the mechanism of the pathogenesis of Tetanus.

TABLE I

Inhibition of Neurite Outgrowth

Inducer	Inhibitors		
	Colchicine	Cytochalasin B	Tetanus Toxin
Minus Serum	Sensitive	Sensitive	Sensitive
Aminopterin	Sensitive	Resistant	Resistant
Bt ₂ cAMP	⁺ Resistant	Sensitive	Resistant
Glial Cell Conditioned Medium	Sensitive	Resistant	Sensitive

TABLE II

Comparison of Kinetics of Total Gross binding

Culture Conditions	t 1/2 ON	K ₁	K ₋₁	K _D
DMEM-FCS	13s	$5.33 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$	$1.92 \times 10^{-4} \text{ sec}^{-1}$	$3.6 \times 10^{-15} \text{ M}$
DMEM + FCS INEFFECTIVE	22.5s	$3.00 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$	$1.92 \times 10^{-4} \text{ sec}^{-1}$	$6.4 \times 10^{-15} \text{ M}$
DMEM - FCS + NAase	252s	$2.75 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	-	-
DMEM - FCS + β -galase	408s	$1.69 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	-	-
GCCM + FCS	360s	$1.925 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	$3.85 \times 10^{-4} \text{ sec}^{-1}$	$2.00 \times 10^{-13} \text{ M}$
GCCM - FCS	255s	$2.717 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	$3.85 \times 10^{-4} \text{ sec}^{-1}$	$1.4 \times 10^{-13} \text{ M}$
Bt ₂ cAMP INEFFECTIVE	385s	$1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	$3.85 \times 10^{-4} \text{ sec}^{-1}$	$2.13 \times 10^{-13} \text{ M}$
Aminopterin INEFFECTIVE	300s	$2.31 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	$3.85 \times 10^{-4} \text{ sec}^{-1}$	$1.66 \times 10^{-13} \text{ M}$

125I-Toxin Binding in Presence of Inducers of Morphological Differentiation

Inducer	Mean * cpm bound	Mean Cell µg Protein	cpm per µg cell protein	µg Toxin per µg Protein	Molecules per Cell
DMEM - FCS	6500	235	27.65	1.58×10^{-6}	3.177×10^3
GCCM + FCS	7500	252	29.76	1.701×10^{-6}	3.421×10^3
GCCM - FCS	5800	178	32.58	1.862×10^{-6}	3.745×10^3
Bt ₂ cAMP	7250	376	19.28	1.102×10^{-6}	2.216×10^3
Aminopterin	6000	265	22.64	1.294×10^{-6}	2.602×10^3

* n = 10 experiments ; Glial cell conditioned medium = GCCM

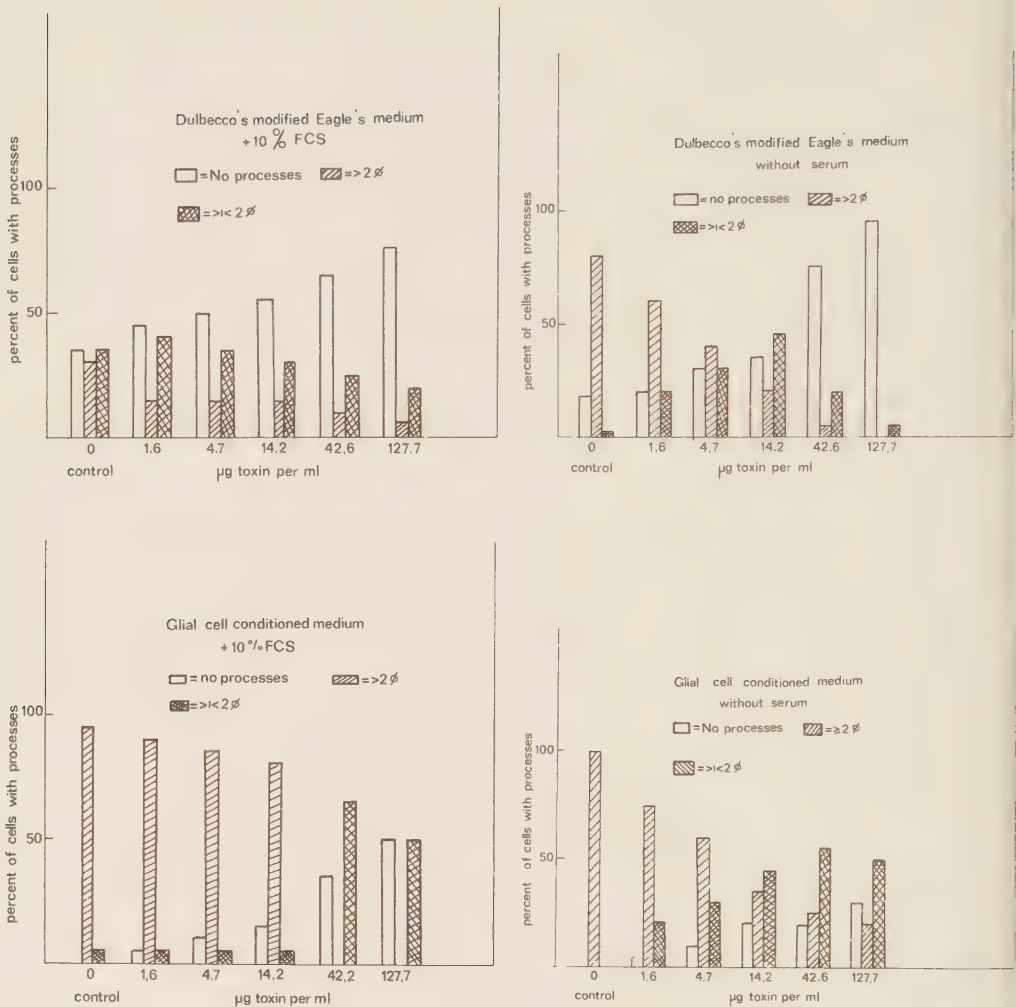


Fig. 1 : Bar graphs represent the percentage of cells having extended neurites after 4 h exposure to high concentrations of tetanus toxin at 37°C :
 a) in DMEM + 10 % FCS, the $\text{RMD}_{504\text{h}} = 1.6 \mu\text{g}$ toxin/ml for the process > 2 cell diameters in length and $48.2 \mu\text{g/ml}$ for total processes.
 b) DMEM - FCS, $\text{RMD}_{50\text{dh}} = 4.7 \text{ ug/ml}$ ($> 2 \phi$) and 18.2 ug/ml ($\geq 1 \phi$).
 c) GCCM + 10 % FCS, $\text{RMD}_{504\text{h}} = 17.8 \mu\text{g/ml}$ ($> 2\phi$) and $127.7 \mu\text{g/ml}$ ($\geq 1\phi$). d) GCCM - FCS, $\text{RMD}_{50} = 9.4 \mu\text{g/ml}$ ($> 2\phi$) and $127.7 \mu\text{g/ml}$ for $\geq 1\phi$.

The dynamics of the reversal are visible in the rise of the cross-hatched column, the intermediate length of processes. The neurites seem to shrink from long to medium, then medium to short. The long ones appear to be much more sensitive to the toxin action

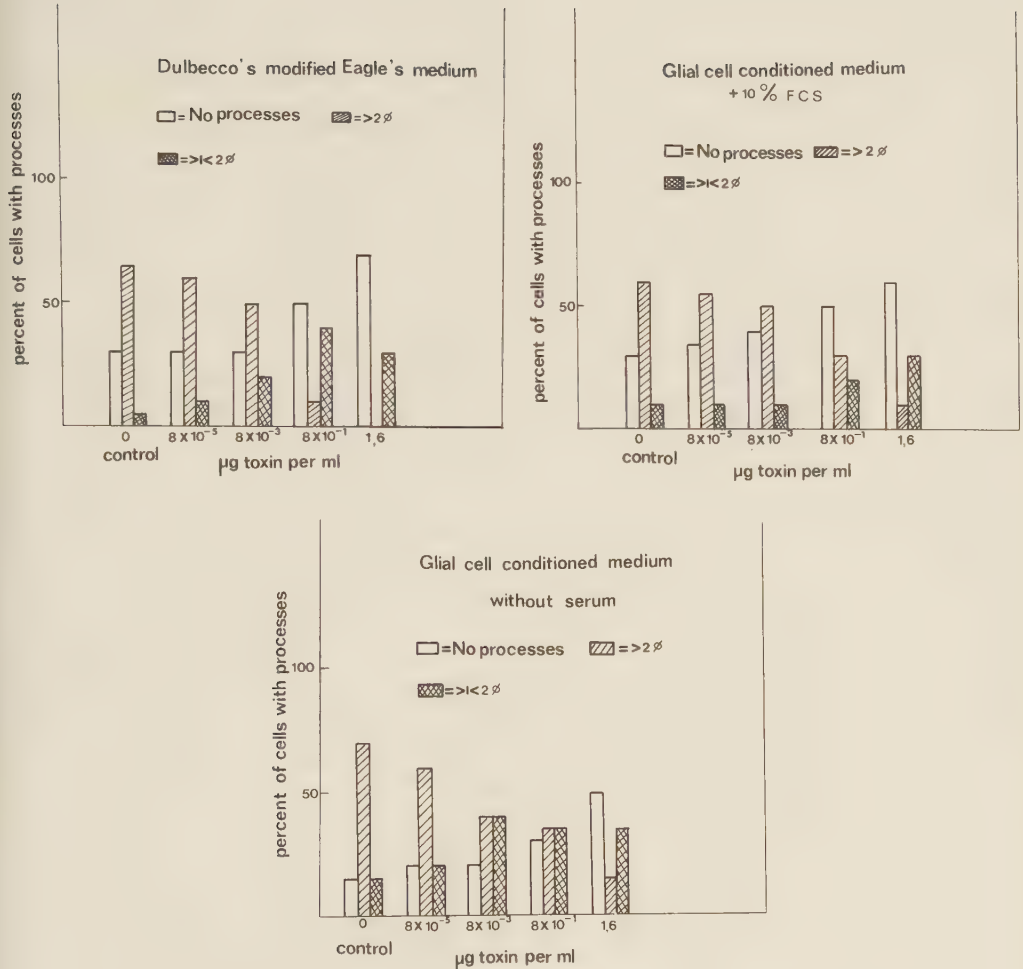


Fig. 2 : Lower concentrations of toxin added to cells and incubated for 24 h.
 a) In DMEM - FCS, at $8 \times 10^{-1} \mu\text{g/ml}$ the $\text{RMD}_{50}24$ is reached for total ($\geq 1\emptyset$) processes ; at about $8 \times 10^{-2} \mu\text{g/ml}$ 50 % of the $> 2\emptyset$ processes are reduced. b) GCM + 10 %, the $\text{RMD}_{50}24 = 8 \times 10^{-1} \mu\text{g/ml}$ is equivalent for all processes. c) GCCM - FCS, the $\text{RMD}_{50}24 =$ about $8 \times 10^{-1} \mu\text{g/ml}$ for $> 2\emptyset$ length processes, and the $\geq 1\emptyset$ cell processes are not reduced 50 % even after 24 hours. In DMEM + 10 % FCS controls, all the cells were round after 24 h.
 We interpret these results to signify that there is a critical internalized concentration of toxin necessary to reverse the morphological differentiation. In presence of the positive stimulus of GCCM, it takes more toxin to force the retraction.

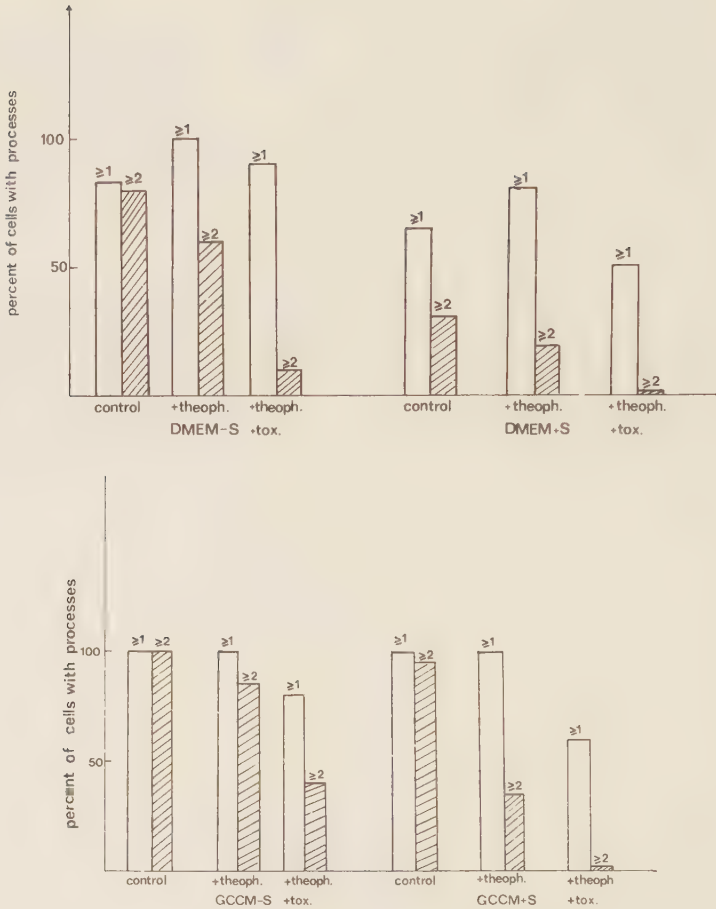


Fig. 3 : Theophylline is known to inhibit cAMP phosphodiesterase at low concentrations. Cells which had been induced to neurite extension with GCCM + 10 % FCS for 48 h were reincubated in presence of DMEM (with and without serum) and GCCM (with and without serum). Tetanus toxin was added to one group of each set along with theophylline. Cells were observed after 4 h. a) In DMEM (+ and - FCS) the theophylline effect was a stimulation of total processes in both cases. The percent of $> 2 \emptyset$ neurites was reduced, but those present were actually much longer than in control cultures. The toxin reduced the long processes by 50 % in absence of serum and ten-fold in presence of serum. b) With GCCM, theophylline reduced the number of $> 2 \emptyset$ processes, to a lesser extent in absence than in presence of serum. Overall neurites were not reduced, hence the GCCM effect. Tetanus toxin reduced both long and short processes, and practically eliminated them in presence of FCS.

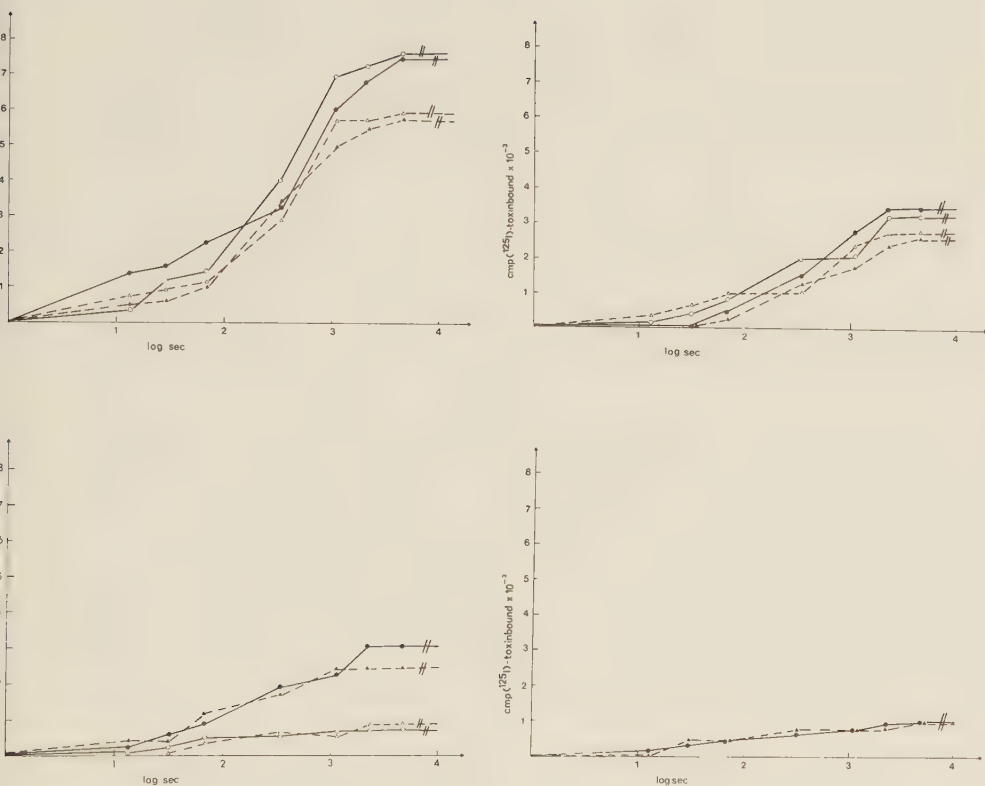


Fig. 4 : ^{125}I -toxin binding in presence of inducers of morphological differentiation. a) total gross binding with $\circ\text{---}\circ$ $\text{Bt}_2\text{cAMP1}$ $\bullet\text{---}\bullet$ $\text{GCCM} + 10\% \text{ FCS}$; $\triangle\text{---}\triangle$ aminopterin; and $\blacktriangle\text{---}\blacktriangle$ $\text{GCCM} - \text{FCS}$. b) native unlabelled toxin. c) binding in presence of neuraminidase. d) association in presence of β -galactosidase.

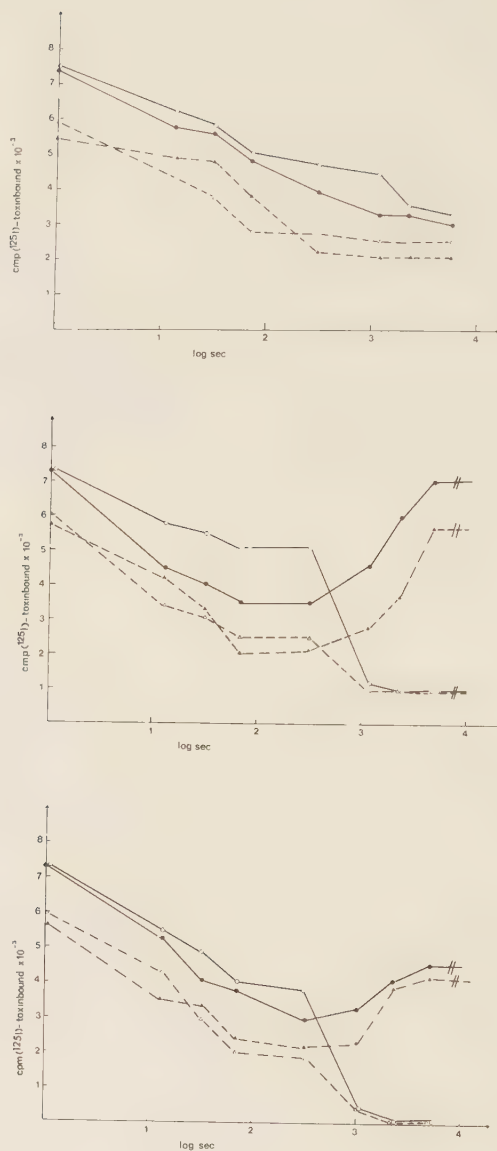


Fig. 5 : Dissociation of bound ^{125}I -tetanus toxin in cultures influenced by ○—○ Bt₂cAMP, ●—● CGGM + 10 % FCS, △—△ aminopterin, and ▲—▲ GCCM - FCS. a) displacement by native toxin, b) removal with neuraminidase, and c) displacement with β -galactosidase. The re-binding in b) and c) in GCCM cultures is associated with EFFECTIVE binding.

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